The Pennsylvania State University
The Graduate School
College of Medicine

INSIGHTS INTO HUMAN NEUTRAL CERAMIDASE TRANSCRIPTION AND THE ROLE OF CERAMIDE METABOLISM IN THE DEVELOPMENT OF AN INHIBITOR OF RESTENOSIS

A Dissertation in
Pharmacology
by
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Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

May 2009
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ABSTRACT

Atherosclerosis is the predominant underlying cause of cardiovascular disease (CVD). CVD is the leading cause of death in the United States and much of Europe, thus the prevention and successful treatment of atherosclerosis is of utmost importance in combating CVD. Atherosclerosis is an inflammatory disease of the arteries where inflammation induces a build up of fat- and cholesterol-laden immune cells and hyperproliferative vascular smooth muscle cells (VSMC). Sphingolipids are naturally occurring lipids with important structural and signaling roles and have identified roles in multiple diseases including atherosclerosis, often due to dysfunctional regulation of their metabolism. The focus of our studies is to better understand the regulation of sphingolipid metabolism and how this may apply to the therapeutic treatment of atherosclerosis.

An important enzyme in sphingolipid metabolism is neutral ceramidase (nCDase). NCDase catalyzes the first step in the conversion of apoptotic ceramide into anti-apoptotic/pro-mitogenic sphingosine-1-phosphate (S-1-P). S-1-P has been reported to induce pro-atherogenic effects on VSMCs. Moreover, inflammatory cytokines involved in the atherogenic process, such as tumor necrosis factor-alpha and interleukin-1beta can induce nCDase transcription and protein expression, leading to increased S-1-P levels. As nCDase could have important ramifications on the progression of atherosclerosis, we chose to study the regulation of nCDase at the level of transcription. Our studies determined the proximal promoter of the nCDase gene and identified multiple functional transcription response elements within the proximal promoter. Furthermore, we determined that the growth factor- and cytokine-activated transcription factor, AP-1,
regulated nCDase transcription. These studies have given us an insight into the transcription regulation of nCDase that may be exploited to control its expression.

Our studies of sphingolipid metabolism also focused on the catabolism of ceramide in a vascular system and its applicability to restenosis therapy in animal models of atherosclerosis. A common treatment for atherosclerosis is angioplasty with, or without, stent implantation, but angioplasty often leads to restenosis, or reclogging of the artery due to hyperproliferation of VSMCs in response to the arterial injury induced by the angioplasty. This body of work extends previous work and shows that cell-permeable C₆-ceramide can limit stenosis in multiple large arterial beds in response to stretch injury. Furthermore, while the ceramide inhibited VSMC growth in vivo and in vitro, vascular endothelial cells (EC) were not as susceptible to the growth inhibitory effects of ceramide and the endothelium was able to wound heal after angioplasty with C₆-ceramide-coated balloon catheters. The role of ceramide metabolism was explored in the differential response of VSMCs and vascular ECs to ceramide treatment. Interestingly, vascular ECs, compared to VSMCs, were found to contain higher levels of ceramide kinase, but not nCDase, and had an enhanced capacity to metabolize the administered ceramide into ceramide-1-phosphate (C-1-P). In addition, we also demonstrated that C-1-P protected cell apoptosis induced by serum-starvation. These studies indicate that the differential metabolism of ceramide by arterial cells makes exogenous ceramide a promising therapeutic for the prevention of restenosis.

The observations described in this dissertation increase our understanding of the transcriptional regulation of an important enzyme, nCDase, in sphingolipid metabolism, which may eventually be used in the design of drugs to regulate nCDase in multiple
diseases including atherosclerosis. Other studies in sphingolipid metabolism reveal that C₆-ceramide has strong potential to be an effective therapeutic in the prevention of restenosis. Collectively, the results of this dissertation emphasize the prospects of exploiting sphingolipids and sphingolipid metabolism in drug design.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>aCDase</td>
<td>Acid ceramidase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>AKT</td>
<td>See PKB</td>
</tr>
<tr>
<td>alkCDase</td>
<td>Alkaline ceramidase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activator protein-2</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATM</td>
<td>Atmosphere</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BASKET</td>
<td>Basel Stent Cost Effectiveness Trial</td>
</tr>
<tr>
<td>C-1-P</td>
<td>Ceramide-1-phosphate</td>
</tr>
<tr>
<td>C₆-C-1-P</td>
<td>C₆-Ceramide-1-phosphate</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CDase</td>
<td>Ceramidase</td>
</tr>
<tr>
<td>CERT</td>
<td>Ceramide transport protein</td>
</tr>
<tr>
<td>Cer</td>
<td>Ceramide</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DES</td>
<td>Drug eluting stents</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median effective dose</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ESI-MS/MS</td>
<td>Electrospray ionization-tandem mass spectrometry</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FOG</td>
<td>Fiend of GATA</td>
</tr>
<tr>
<td>Fr</td>
<td>French</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCS</td>
<td>Glucosylceramide synthase</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Process</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
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<td>GTF</td>
<td>General transcription factors</td>
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<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HCAEC</td>
<td>Human coronary artery endothelial cells</td>
</tr>
<tr>
<td>HCASMC</td>
<td>Human coronary artery smooth muscle cells</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>JDP</td>
<td>Jun dimerization protein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH$_2$ terminal kinase</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MCSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>nCDase</td>
<td>Neutral ceramidase</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NOE</td>
<td>N-oleylethanolamine</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand white</td>
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O^-  Superoxide anion
ORF  Open reading frame
oxLDL  Oxidized low density lipoprotein
QRT-PCR  Quantitative real-time polymerase chain reaction analysis
PBS  Phosphate buffered saline
PDGF  Platelet derived growth factor
PKA  Protein kinase A
PKB  Protein kinase B
PKC  Protein kinase C
PTA  Percutaneous transluminal angioplasty
PTCA  Percutaneous transluminal coronary angioplasty
QCM-D  Quartz Crystal Microbalance with Dissipation
RNAi  RNA interference
ROS  Reactive oxygen species
rS1P  Sphingosine-1-phosphate receptor
RT-PCR  Reverse transcriptase PCR
S-1-P  Sphingosine-1-phosphate
siRNA  small interfering RNA
SMase  Sphingomyelinase
SMC  Smooth muscle cell
SMD  Structured membrane microdomain
SPT  Serine palmitoyl transferase
Sph  Sphingosine
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SVG</td>
<td>Saphenous vein graft</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TESS</td>
<td>Transcriptional Element Search Software</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TRE</td>
<td>Transcriptional response element</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
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<tr>
<td>µmol</td>
<td>Micromole</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
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Preface

Chapter 3 of my dissertation is derived from published data that I am first author for. However, the data is a result of multiple-authored work. In this section, I would like to acknowledge the other contributing authors and specify which data each author contributed. I will address the figures derived from the work of the other authors in order of how they are arranged in chapter 3.

The data shown in Figure 3.1A and quantified in Figure 3.1B-C were the results of work overseen by Linda Karavodin from REVA Medical, Inc., San Diego, California and performed at Lychron, Inc., Mountain View, California. The data shown in Figure 3.1E-F and quantified in Figure 3.1D were the results of the combined work of Ronald Wilson, Mark Kozak, and Peter Waybill from Penn State College of Medicine. The data pictured in Figure 3.1G were achieved from the efforts of Ronald Wilson and Tony Brown from Penn State College of Medicine.

Dina Olympia from Penn State College of Medicine assisted in experiments yielding results displayed in Figure 3.2A-C.

Todd Fox assisted with the mass spectrometry analyses of lipids displayed in Table 3.1.

The data on fibrinogen absorption depicted in Figure 3.5A-B was provided by Norbert Weber at Rutgers University.

The data shown in Figure 3.6A-C were obtained through the efforts of Dina Olympia and Kristy Houck from the Penn State College of Medicine. Todd Fox, Tom Stover, Tony Brown, and Ronald Wilson from Penn State College of Medicine took part in the animal experiments depicted in Figure 3.6D-F. The histochemical analyses of
these experiments were performed by Steve Levinson while he as at the Penn State
College of Medicine.

The pharmacological and toxicology analyses depicted in Table 3.2 and described
in the text were performed under the direction of Linda Karavodin from REVA Medical,
Inc.

I would like to thank each of the aforementioned scientists for the help they gave and
work that they did, which more than helped me in the completion of this dissertation.
Acknowledgments

I would like to dedicate this body of work to my father, Ronald O’Neill. RIP, April 23, 1939—December 27, 2005. There could be no one more proud of me than he would be as I complete my graduate career, and it pains me that he is not here to share the culmination of my schooling with me. I cannot thank him enough for being the wonderful father that he was, and the loving memories of him remain eternal in my heart.

I would like to thank, first and foremost, the members of my committee, Mark Kester, JongYun, Bogdan Prokopczyk, Cara Schengrund, and Mel Billingsley, for their guidance and ideas throughout the development of my research. I especially would like to express my gratitude towards Mark Kester, who not only served as my thesis advisor, but also as my mentor as I matured throughout the years into a young scientist. Mark was someone who believed in me and was very patient as I developed both personally and scientifically, and for this, I am forever grateful. I could not have asked to work for a more flexible and understanding person, and because of him, I enjoyed my years of training and research in the pursuit of my degree. In addition, I would like to thank Jong Yun for the extra attention and help that he gave me over the course of my work.

Also, I would like to thank the Kester Lab, both past and present, for all their help in my studies and for helping create a healthy work environment that made it desirable to come to work everyday. In particular, I’d like to thank Kristy Houck, Todd Fox, Tony Brown, James Kaiser, Onur Unal, Dina Olympia, Tom Stover, Nikki Hoffman, Yasser Heakel, Sindura Ganapathi, and Brian Barth.
I would like to acknowledge the Proteomics Core Facility of the Section of Research Resources, Penn State College of Medicine for their aid in many of my experiments, both through their knowledge and the use of their scientific instruments.

I would also like to thank my family for their support through the many difficult times that I endured throughout my graduate career. My brothers Ryan and Craig are my best friends, and they made it possible to get through school despite the adversities that we have dealt with over the past few years. I also appreciate the moral and monetary support received from my mom and stepfather, Donna and Jerry Smith, and from my father and stepmom, Ronald and Kitch O’Neill.
Chapter 1

Literature Review
1.1 Introduction

Lipids are an important class of biomolecules that are broadly defined as fat-soluble (lipophilic) molecules. Lipids are biologically important molecules for energy storage and are critical structural components of cellular and organelle membranes. Human diseases resulting from defects in lipid metabolism reveal that lipids have crucial roles in the physiology of organs down to cells. Over the past couple of decades, research has demonstrated that lipids are important mediators in cellular signal transduction pathways. However, studies in the field of lipidomics have lagged far behind that of genomics and proteomics mostly because of lipid complexity and the lack of sensitive tools to study lipids. Recently, the field of lipidomics has finally begun to grow at an accelerated pace because of advances in mass spectroscopic techniques such as electrospray ionization and matrix-assisted laser desorption/ionization (1). With these advances, it is now possible to elucidate the role of lipids in many diseases including hypertension, atherosclerosis, coronary heart disease, Alzheimer's disease, schizophrenia, obesity, diabetes mellitus, and cancer (2). Our laboratory is interested in a specific class of lipids, i.e., sphingolipids, and their role in atherosclerosis and cancer. In particular, we are interested in the role of sphingolipid metabolites and sphingolipid metabolizing enzymes in the etiology and treatment of atherosclerosis and the prevention of restenosis.

1.2 Atherosclerosis

Cardiovascular disease (CVD) ranks as one of the world’s deadliest diseases. In the mid 1990’s, cardiovascular disease was the principle cause of death in the United States (US), Europe, and much of Asia (3, 4). A decade later, CVD is still a leading
cause of death with one third of yearly deaths attributed to it (5). The predominant underlying cause of CVD, including ischemic stroke, coronary heart disease (CHD), and acute coronary syndromes (ACS), is atherosclerosis (6). Atherosclerosis, simply stated, is a condition in which there is a build up of plaque, consisting of fat, cholesterol, calcium, and other substances, within arterial beds. Some of the most grave complications of atherosclerosis result when the coronary arteries are affected, resulting in coronary artery disease (CAD). In the US, the instances of CAD have been on a decline since the 1970’s due to a decrease in smoking and lowering of blood low-density lipoprotein (LDL) levels via diet and pharmaceutical management; however the rate of decline in recent years has been decreasing due to an aging US population and the growing obesity epidemic (7).

Atherosclerosis is an inflammatory disease in which there is a progressive and dynamic build up of lipids, inflammatory cells, connective tissue elements, smooth muscle cells (SMCs), thrombi, and calcium deposits, collectively referred to as plaque (8, 9) (Fig. 1.1). The accepted hypothesis is that endothelial cell dysfunction of the arterial endothelium is the first step in atherosclerosis (10). Several factors that lead to endothelial cell dysfunction are, but not limited to, elevated modified LDL, cigarette smoke-induced free radicals, hypertension, and diabetes mellitus (11). Endothelial cell dysfunction preferentially occurs at sites within the arteries where there are decreases in shear stress or increased turbulent flow (8, 12). The dysfunction induces the endothelium to have pro-coagulant properties as opposed to anticoagulant properties (10). LDL particles are responsible for the initial inflammatory response, whereby they infiltrate a
dysfunctional endothelium and accumulate at sites of hemodynamic strain on the intima of the artery (9).

**Figure 1.1 An overview of the progression of atherosclerosis.** Modified from the source Dzau et. al. (13). Atherosclerosis is an inflammatory disease of the vasculature that is characterized by a progressive and dynamic build-up of lipids, inflammatory cells, connective tissue elements, smooth muscle cells (SMCs), thrombi, and calcium deposits, collectively referred to as plaque. Disruption of the endothelium by atherogenic factors can cause an inflammatory response that, if not quickly subdued, can progress to the formation of a fatty streak. Continued plaque progression and the release of inflammatory mediator can weaken the cap of the lesion and allow the plaque to rupture and cause thrombotic events. If the cap is thicker, the plaque can continue to grow as a stable plaque and subsequently cause a narrowing of the lumen. Abbreviations represented in the figure are as follows: TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin 1, LDL, low-density lipoprotein; MCP, monocyte chemoattractant protein; VCAM, vascular cell adhesion molecule; EGF, epidermal growth factor; IFN, interferon; PDGF-BB, platelet-derived growth factor-BB, IGF, insulin-like growth factor; and finally, bFGF, basic fibroblast growth factor.
LDL particles, specifically, the trapped LDL particles at the intima, can be progressively oxidized to form oxidized LDL (oxLDL). The oxLDL at the intima upregulates the genes for macrophage colony-stimulating factor (MCSF) (5) and monocyte chemotactic protein-1 (MCP-1) in arterial endothelial cells (14, 15). Therefore the oxLDL promotes an inflammatory response by causing the chemotaxis of inflammatory monocytes and inducing their maturation into mature macrophages. Furthermore, oxLDL and hemodynamic stress induce the expression of leukocyte adhesion molecules such as selectins, intercellular adhesion molecules (ICAM), and vascular-cell adhesion molecules (VCAM), which promote the adherence, migration, and accumulation of monocytes and T-cells (9, 10).

The monocyte-derived macrophages and T-cells mediate the inflammatory response throughout all stages of atherosclerosis (16). The activated macrophages and T-cells secrete cytokines such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and MCSF, which increase the binding of LDL to the endothelium and increase the transcription of the LDL-receptor gene (17, 18). The oxLDL induces scavenger receptors on the surface of macrophages (19), which in turn, recognize the oxLDL and allow the macrophage to internalize oxLDL (20). Internalization of oxLDL and cholesterol transforms the macrophages into foam cells. Deposition of the lipid-laden foam cells on the intima leads to the formation of a fatty streak, the first structural change observed in atherosclerosis (12).

If the initial “protective” inflammation response by the macrophages and T-cells does not neutralize or remove the inflammatory agents, the response continues and eventually stimulates the SMCs in the medial layer to migrate to the fatty streak at the
intima. The SMCs then proliferate and intermix at the site of inflammation. SMCs at the inflammatory lesion produce extracellular matrix proteins like collagen and elastin (21), eventually leading to the formation of an intermediate lesion, or fibrous plaque (9, 12, 21). The combination of SMCs and extracellular matrix actually form a fibrous cap over a core of lipid and necrotic tissue. The continued release of hydrolytic enzymes, cytokines, chemokines, and growth factors from the activated macrophages and T-cells induces focal necrosis within the plaque (10). The core is thrombogenic but is protected from circulating platelets and coagulation proteins by the fibrous cap (12). Erosion of the endothelium or plaque rupture can expose the plaque leading to thrombosis or increased inflammatory activity through macrophage/oxLDL interactions (12, 21). Repeated cycles of erosion or rupture, thrombosis, and subsequent repair gradually increase the size of the plaque.

The current view is that thrombosis may be more clinically detrimental than the stenosis that occurs from the growing plaque. The fibrous caps of many plaques are relatively thin, thus they may have a high propensity to rupture and expose the thrombogenic core. Such types of plaque are referred to as vulnerable plaques. Myocardial ischemia or infarction is believed to occur more frequently from vulnerable plaque rupture and thrombus formation as compared to narrowing of the lumen from plaque build up (9). Without using invasive methodologies, vulnerable plaque rupture and thrombus formation are difficult to detect and stop before injury has occurred. Multiple investigations are focused on developing non-invasive technologies that could be used to “visualize” atherosclerotic lesions, in order to identify and treat developing plaque before significant thrombotic events occur. On the other hand, the complete
blockage of an artery from stenosis is a slow process and symptoms usually become apparent before significant damage has resulted. The continued growth of plaque causes a thickening of the artery wall; however arteries will initially compensate for this gradual thickening with gradual dilation to maintain the lumen size, a process known as “remodeling” (22). Only after the artery can no longer remodel itself will the lumen diameter start to narrow with continued plaque build up. Plaque buildup can often be detected before complete blockage of the artery occurs and before drastic ischemic or infarct injury occurs. Plaque blockage can be circumvented by bypass surgery for severely blocked arteries or corrected via angioplasty with or without stents for less severe blockage.

Angioplasty is a very effective procedure for opening up the lumen of arteries that have been narrowed from plaque build up. Basically, angioplasty is a process where a deflated balloon catheter is inserted through the artery to the area of plaque build-up and inflated to stretch the artery and compress the plaque. This process increases the diameter of the lumen, thus reestablishing better blood flow. One problem with this procedure is that up to 40% of patients develop restenosis (23), whereby the lumen of the artery becomes narrow again due to smooth muscle cell hyperplasia and collagen deposition (24). Use of stents, or scaffolds, to support the angioplastied artery significantly reduce the occurrence of restenosis, but 10% to 40% of patients, depending on their individual circumstances, still have in-stent restenosis as a result of intimal hyperplasia (25). In-stent restenosis has been managed by the direct delivery of a therapeutic agent using drug eluting stents. The drugs paclitaxel and sirolimus are currently used in drug-eluting stents and inhibit smooth muscle cell growth by preventing
microtubule disassociation and inhibiting mTOR, respectively (26). However use of paclitaxel and sirolimus on stents has been implicated in delayed or absent endothelialization of angioplasty-traumatized arteries, possibly resulting in coronary endothelium dysfunction (27, 28). Interestingly, with the use of drug-eluting stents to prevent restenosis, incomplete reendotheliazation is a primary predictor of thrombotic events (29, 30). Therefore, the development of drugs that can inhibit smooth muscle growth, yet allow reendothelialization, would have the therapeutic potential to limit restenosis in angioplastied or stented arteries.

1.3 Sphingolipids

Sphingolipids make up a complex, ubiquitous class of lipids. The name “sphingolipid” is derived from “Sphinx,” a mythological creature with the head of a human and the body of a lion, due to the enigmatic properties that arise from the sphingolipid backbone containing both an alipathic group and an amino alcohol. Within mammals, the defining “sphingoid” backbone of sphingolipids is sphingosine (see Fig.1.2). The structural role of sphingolipids in cellular membranes has long been known. Some functions of sphingolipids in the plasma membrane are increasing the membrane’s mechanical stability and, also, chemical resistance. However over the past two decades, these enigmatic sphingolipids have been identified as intracellular signal transducing molecules, and it is now well-accepted that sphingolipids and their metabolic enzymes have critical roles in cell signaling.
Figure 1.2 Sphingolipid metabolic pathways. Sphingolipids are defined by the presence of a sphingoid backbone known as sphingosine. However, ceramide is the central molecule in sphingolipid metabolism. It is synthesized through a de novo pathway, initiated by the rate limiting condensation of serine with palmitoyl-CoA. Ceramide then can be converted directly, or through a series of enzymatic reactions, into many other sphingolipid species as pictured.
Although the D-erythro-sphingosine moiety defines the backbone of sphingolipids, ceramide is the central molecule in sphingolipid metabolism, and the majority of other sphingolipids are derived from additions to the ceramide molecule (Fig. 1.2). Ceramides contain a sphingosine backbone which has had one of several chain length fatty acids attached to it by an amide bond at the 2-amino position. Complex sphingolipids are formed by the addition of hydrophilic head groups to the OH-group at the C-1 position. The hydrophilic head groups could be sugar groups or phosphorylcholine in the case of glycosphingolipids (GSLs) or sphingomyelin, respectively. These substitutions at both the 2-amino and 1-hydroxyl positions allows for the formation of a plethora of sphingolipid species.

As mentioned, sphingolipid metabolism revolves around ceramide and there is much recycling of sphingolipids from one metabolite to the next. However, sphingolipid metabolism initially starts with the *de novo* synthesis of ceramide. *De novo* synthesis of ceramide is initiated by serine palmitoyl transferase (SPT) as it catalyzes the condensation of serine and palmitoyl-CoA in the rate limiting step of ceramide synthesis (31). Three additional enzymes facilitate the complete synthesis of ceramide at the outer leaflet of the endoplasmic reticulum (ER) membrane, completing the *de novo* synthesis of ceramide (Fig 1.3). Within the ER, ceramide can be used as a substrate by galactosylceramide synthase to synthesize galactosylceramide (32, 33), whereas ceramide must be exported out of the ER for the synthesis of most other sphingolipids.
Figure 1.3 Intracellular localization of sphingolipid metabolism.
Adapted from the source Hannun and Obeid, 2008 (34). Many of the subcellular localities of sphingolipid metabolism have been identified. The \textit{de novo} synthesis of ceramide occurs at the ER before it is transported to the Golgi to be converted into more complex sphingolipids. The catabolism of complex sphingolipids to ceramide and of ceramide to sphingosine occurs preferentially in lysosomes where acidic enzymes reside, but also at the plasma membrane where neutral enzymes reside.
Export of ceramide out of the ER for further sphingolipid synthesis occurs via at least two distinct processes depending on the final sphingolipid product (Fig. 1.3). The first pathway is a vesicular-independent pathway, in which ceramide is actively transported to the Golgi apparatus via the ceramide transport protein (CERT) (35). CERT-transported ceramide is used solely as a substrate for sphingomyelin synthase (35), whereby a phosphocholine headgroup from phosphatidylcholine is added to the ceramide to synthesize sphingomyelin (36). Ceramide that is to be synthesized into glucosylceramide and subsequent more complex sphingolipids is brought to the Golgi outer leaflet by vesicular transport where glucosylceramide synthase (GCS) can add a glucose moiety to the ceramides (37, 38). Glucosylceramide is translocated to the Golgi lumen where it can be synthesized into lactosylceramide and subsequent GSLs (39).

In contrast to synthesizing sphingolipids by building upon ceramide, certain important sphingolipids are the products of catabolizing ceramide. The varying fatty acid side chains of ceramide can be removed by hydrolysis, catalyzed by one of multiple ceramidases (CDases), leaving sphingosine as a product (40, 41). Sphingosine is either recycled back into the sphingolipid metabolic pathway or sphingosine kinase can phosphorylate it, creating sphingosine-1-phosphate (S-1-P) (42). S-1-P can be broken down by S-1-P lyase into non-sphingolipid products, thus providing an “exit-point” from the sphingolipid metabolic pathway (43).

For the most part, excluding the breakdown of S-1-P, the reactions discussed above that generate the various sphingolipids from ceramide are all reversible. The reverse reactions are catalyzed by enzymes distinct from the respective forward reaction enzymes, thus all leading to ceramide generation. So as opposed to de novo synthesis,
ceramide can also be generated catabolically by the breakdown of other sphingolipids. For some time now, the generation of ceramide from sphingomyelin via removal of the phosphocholine by sphingomyelinases (SMases) has been shown to be critical in sphingolipid signaling (44). More recently, the conversion of sphingosine into ceramide via ceramide synthases in the “salvage” or recycling pathway has become an apparent facet of sphingolipid signaling (45). There has been a wealth of information produced over the past two decades about the participation of sphingolipids in cellular signaling. It is now well accepted that sphingolipids have crucial roles in regulating biological functions including, but not limited to, cell proliferation, cell cycle arrest, apoptosis, differentiation, and migration (46, 47, 48). Pioneer work in sphingolipid bioactivity elucidated the importance of the ceramide to sphingosine to S-1-P transition in modulating signal transduction. Ceramide was shown to regulate responses to cellular stress such as growth arrest, apoptosis, and cell senescence (49, 50). Contrarily, S-1-P has a potential role in cell survival, proliferation, migration, and inflammatory pathways (47). For years attention was focused on ceramide and S-1-P, however, a new collection of data has been acquired implicating other sphingolipids as having important biological functions. For instance, ceramide-1-phosphate (C-1-P) has been shown to have a role in inflammation, proliferation, and vesicular trafficking (51, 52, 53). Glucosylceramide is able to facilitate post-Golgi trafficking and affect drug resistance (54, 55). Moreover, the pathological roles of complex GSLs in many disease states, such as diabetes, have been identified in recent years. Extensive research continues to be done not only on ceramide, sphingosine, and S-1-P, but also on the many other identified sphingolipids, furthered by
sophisticated LC/MS/MS (liquid chromatography/mass spec/mass spec) lipodomic analyses and quantification.

1.4 Sphingolipids and Atherosclerosis

The crucial roles that sphingolipids play in cellular signaling are important in disease states such as atherosclerosis. In addition to participating in signaling cascades, sphingolipids can also influence the atherogenic process by affecting lipoprotein metabolism. Multiple sphingolipid species have known associations with atherosclerosis, having both atherogenic and non-atherogenic roles. The development of atherosclerosis correlates with increased concentrations of sphingomyelin in blood plasma, and hence sphingomyelin levels are one of several independent predictors of CAD (56). Interestingly, in a mouse model of atherosclerosis, feeding mice a high sphingolipid-rich diet resulted in an increase of aortic atherosclerotic lesions (57). As mentioned earlier, high LDL levels are strongly atherogenic and, therefore, have critical roles in the initiation of atherogenesis. Contrarily, high density lipoproteins (HDL) are strongly anti-atherogenic and are able to sequester cholesterol away from arteries to the liver for proper cholesterol metabolism. Different sphingolipid species associate with lipoproteins and the amount associated with a particular lipoprotein can depend on the disease state. LDL isolated from atherosclerotic lesions contains significantly higher levels of sphingomyelin compared to plasma LDL (58, 59). The ceramide content in LDL, specifically aggregated LDL, in atherosclerotic lesions was 10-50 fold higher than ceramide levels in plasma LDL, thus implicating ceramide in LDL aggregation (60). These data suggested that atherogenic lipoproteins, i.e., LDL, are able to deliver sphingomyelin to the arterial
wall where SMases can metabolize sphingomyelin into ceramide. The increased ceramide levels promote LDL aggregation and subsequent foam cell formation (60, 61). Glycosphingolipids are predominantly associated with LDL (62). Glucosylceramide was the major GSL found in human intimal atherosclerotic plaque, although there was an appreciable amount of lactosylceramide present also. Notably, both of these GSLs were not present in the non-lesioned intima (63). Differing from ceramide and GSLs, S-1-P concentrations are much higher in anti-atherogenic HDL relative to S-1-P in LDL (64). Whether or not S-1-P associated with the HDL contributes to the anti-atherogenic effects of HDL remains debatable and its discussion is forthcoming.

Analyses of atherosclerotic plaque from animal models of atherosclerosis revealed significantly higher levels of ceramide within the plaques relative to normal areas of the vascular wall (65). Ceramide is potentially involved with many biological functions related to the pathogenesis of atherosclerosis. TNF-α is a potent pro-inflammatory cytokine that actively participates in the progression of atherosclerosis (66). Treatment of vascular endothelial cells with TNF-α increased intracellular ceramide levels via de novo synthesis (67, 68). TNF-α has also been shown to activate SMases to generate ceramide, followed by the production of reactive oxygen species (ROS) such as superoxide anion (O−) (69). Interestingly, ceramide can induce endothelial cells to express TNF-α (70), which, plausibly, could lead to a cyclic relationship of TNF-α—ceramide—TNF-α production, thus potentiating the damaging inflammatory effects. In addition, ceramide has direct inflammatory effects via promoting interleukin-6 (IL-6) production and subsequently C-reactive protein (CRP) production, thus potentially contributing to the atherogenic process (71). Another independent risk factor for
atherosclerosis, homocysteine, was reported to be deleterious, possibly by activating ceramide synthase and increasing ceramide levels (72, 73, 73, 74). Ceramide is also indirectly produced by highly atherogenic ox-LDL, through activation of matrix metalloproteinase-2 (MMP-2) (75). Ceramide production can be atherogenic through its ability to negatively affect nitric oxide (NO) bioavailability. NO is a critical regulator of vascular homeostasis, and a loss of NO bioavailability through decreased nitric oxide synthase activity or degradation of NO by ROS is a primary cause of endothelial cell dysfunction. Evidence suggests ceramide-induced reduction of NO bioavailability to endothelial cells is mediated through increase in O’ levels (76, 77, 78, 79). Yet, mounting evidence now supports that ceramide metabolizing enzymes may ultimately contribute to the atherogenic phenotype.

Ceramide is not the only sphingolipid that affects NO production. S-1-P can also affect NO production, usually in a receptor-dependent manner. The predominant sphingosine-1-phosphate receptors (S1Pr) in the cardiovascular system are S1Pr-1, -2, and -3 (80, 81, 82). S-1-P is able to increase NO production in endothelial cells through activation of S1Pr-1 and S1Pr-3, which are the predominant S1Pr’s expressed in endothelial cells (83). As S-1-P is found within HDL, it is not surprising that HDL increases NO availability and inhibits oxLDL-induced decreases in NO bioavailability (84, 85). Although results from an S1Pr-3 knockout model suggested HDL effects on NO availability are mediated by S-1-P (86), other data suggested that HDL effects on NO are independent of S-1-P, as HDL lacking S-1-P was able to increase NO production (87). Regardless, the S-1-P-induced NO production by endothelial cells is anti-atherogenic.
On the other hand, S-1-P also has the potential to be pro-atherogenic. A critical inflammatory step in atherosclerosis is monocyte recruitment and adhesion to the endothelium, which is regulated in part by the expression of endothelial cell adhesion molecules. The majority of studies show S-1-P increases adhesion molecule expression in endothelial cells (88, 89). The pro-inflammatory factors TNF-\(\alpha\) and oxLDL both induce adhesion molecule expression by activating sphingosine kinase and increasing S-1-P production (88, 89, 90). Contrary to S-1-P, HDL inhibits adhesion molecule expression, which is consistent with its anti-atherogenic properties (91, 92). An explanation for the apparent contradiction between the effects of HDL and S-1-P was recently proposed in which HDL would act more as a scavenger of S-1-P, rather than a carrier of S-1-P (93). However, the anti-atherogenic and cytoprotective effects of S-1-P on proliferation, migration, and survival of endothelial cells are proposed mechanisms, at least in part, by which HDL prevents oxLDL-induced inhibition of endothelial cell proliferation, migration, and apoptosis (94, 95, 96). S-1-P induces endothelial cell migration through S1Pr-1 and S1Pr-3 and a Rac-1-dependent pathway, where Rac-1 is a critical factor in cytoskeletal rearrangement during cell migration (97, 98). Similarly, S1Pr-1 and S1Pr-3 mediate S-1-P-induced proliferation of endothelial cells; however in this case, the effects are mediated through an extracellular signal regulated kinase (ERK) pathway (82, 97).

The response of vascular smooth muscle cells (VSMCs) to S-1-P can be a little more complicated because of its receptor expression patterns. Whereas endothelial cells express very little or no S1Pr-2, it is the predominant receptor expressed in VSMCs along with moderate levels of S1Pr-3 (80, 82). VSMCs can also express S1Pr-1, but at lower
levels than the other two (80, 82). S-1-P signaling through the S1Pr-2 receptor inactivates Rac-1 leading to inhibition of VSMC migration, thus an anti-atherogenic response. However, signaling through the other two receptors can lead to atherogenic VSMC migration through Rac-1 activation (82), indicating that the migratory properties of VSMC in response to S-1-P are dependent on the receptor subtype expression pattern. A similar situation exists for S-1-P induced proliferation of VSMC. Activation of S1Pr-1 by S-1-P is atherogenic and leads to proliferation in VSMCs expressing relatively higher levels of S1Pr-1 (82). Interestingly, in VSMC that predominantly express S1Pr-2 and S1Pr-3, S-1-P induces DNA synthesis, but, according to most evidence, this probably does not translate to VSMC proliferation (82, 97). These studies reveal that S-1-P has a complex and ambiguous role in the initiation and progression of atherosclerosis. The anti-atherogenic properties of S-1-P tend to be mediated through S1Pr-2 and/or S1Pr-3 in VSMCs, whereas the atherogenic properties are mainly mediated through S1Pr-1. In endothelial cells, S-1-P has both atherogenic (increased adhesion molecule expression) and anti-atherogenic (increase proliferation and migration) effects mediated through S1Pr-1 and S1Pr-3. It is of interest that signaling networks triggered by S1Pr-1/2/3 vary due to different G-proteins coupled to the receptors. Therefore, the overall anti- or pro-atherogenic effect of S-1-P on the vasculature is, at least partially, dependent on the receptor expression profile of the vascular cells.

In addition to ceramide and S-1-P, GSLs may play a role in the development of atherosclerosis. Patients homozygous for familial hypercholesterolemia show 2- to 4-fold elevated levels of GSLs. Patients with hypercholesterolemia and atherosclerosis can have increased GalT-2 (glucosylceramide galactosyltransferase, or rather, the enzyme
that catalyzes lactosylceramide formation) activity and increased lactosylceramide levels (99, 100). At a molecular level, atherogenic oxLDL activated GalT-2 leading to increased lactosylceramide levels, sufficient to induce VSMC proliferation (101, 102). In VSMCs, lactosylceramide generated superoxide release, which activated a mitogen activated protein kinase (MAPK) pathway leading to proliferation (103, 104). Another important role in atherosclerosis is the ability of lactosylceramide generated superoxide to induce expression of cell adhesion molecules on neutrophils and endothelial cells, thus participating in the initiation of neutrophil adhesion to endothelial cells (105, 106). It is also possible that the lactosylceramide-generated superoxide could have other atherogenic effects, such as scavenging NO and depleting NO bioavailability.

The biological properties that sphingolipids exert on the vasculature offer mechanisms by which to exploit them therapeutically. As such, the use of exogenously delivered C₆-ceramide has shown promise as an effective therapy against restenosis, or neointimal hyperplasia, that often arises after initial surgical treatment of atherosclerosis. As mentioned earlier, neointimal hyperplasia at the site of angioplasty is a common, yet deleterious, complication arising from surgical intervention of atherosclerosis due to excessive proliferation of VSMC in response to the vascular injury. Our lab has demonstrated that C₆-ceramide coated onto balloon catheters significantly reduces neointimal hyperplasia induced by angioplasty in rabbit carotid arteries in vivo (107). Additionally, our lab demonstrated that C₆-ceramide downregulates both the mitogenic ERK pathway and the pro-survival AKT pathway, thus leading to VSMC growth arrest (107, 108), and subsequent inhibition of neointimal hyperplasia. Moreover, new evidence suggests that ceramide may, in fact, be anti-inflammatory, possibly by
inhibiting Toll-like receptor 4 signaling cascades (109). The identification of anti-inflammatory sphingolipid metabolites that decrease vascular SMC proliferation and increase endothelial wound healing responses would be viewed as a therapeutic approach for the treatment of restenotic or atherosclerotic lesions. Thus, an understanding of enzymes such as CDase and ceramide kinase is critical for comprehending the role of sphingolipids in the pathogenesis and therapeutic management of atherosclerosis.

1.5 Ceramidases

In the de novo pathway of sphingolipid biosynthesis, there are no enzymes that directly convert dihydrosphingosine to sphingosine. Sphingosine is originally derived from the hydrolysis of ceramide by N-acylsphingosine amidohydrolases, otherwise known as CDases. In addition, phosphorylated sphingosine can be dephosphorylated by sphingosine-1-phosphate phosphatase back into sphingosine. Furthermore, S-1-P formation is highly dependent on ceramide-generated sphingosine. Therefore, CDases maintain a critical role in controlling the balance of ceramide, sphingosine, and S-1-P levels. To date, five mammalian CDases have been identified and cloned. These five CDases are classified into three categories, acidic, neutral, and alkaline, based on the optimal pH for their respective maximum activities.

Acid CDase (aCDase) was the first discovered CDase, and as its name suggests, it is catalytically active at low pH’s. In mammals, both human and mouse acidic CDases have been identified and cloned. The human aCDase gene is located on chromosome 8 (110), and the mouse gene is located on mouse chromosome 8 (111). Both CDases are very similar. The human form is synthesized as a 395 amino acid (AA) single
polypeptide, which is self-cleaved into a heterodimeric enzyme with α and β subunits. Mouse aCDase is also a heterodimeric enzyme with 394 AAs (111). Within cells, aCDase localizes within lysosomes where the pH is low enough to sustain its activity (111, 112). In addition, mouse aCDase was shown to be secreted extracellularly (113). The expression profile of human aCDase was not determined, however mouse aCDase was ubiquitously expressed having high expression levels in the kidney, lung, heart, and brain, but low levels in the spleen, skeletal muscle, and testes (111). The preferred substrates for aCDases are medium acyl chain length ceramides (medium-chain ceramides) such as C₁₂-ceramide (114, 115).

The biological importance of aCDase was first noticed in studies of the genetic disorder Farber’s disease. Farber’s disease is a lysosomal storage disorder resulting from a deficiency of aCDase activity. This results in an accumulation of sphingolipids within lysosomes. The pathology of Farber’s disease originally revealed the critical role aCDase plays in ceramide catabolism. Results from early studies of Farber’s disease patients suggested that human aCDase may only have a little, or no, role in regulating cellular responses. Studies of lymphocytes and fibroblasts obtained from Farber disease patients were as sensitive to various ceramide-inducing stress stimuli including staurosporine, the anticancer drugs etoposide and doxorubicin, and gamma-irradiation, as normal lymphocytes (116). However, more recent studies have suggested that aCDases do indeed play a role in regulating cellular responses. Altering aCDase within cells can have significant effects on apoptosis induced by various stressful stimuli. Knocking down aCDase with RNA interference (RNAi) in head and neck tumor cells in which aCDase was upregulated increased the tumor cells’ susceptibility to anti-Fas receptor antibody
induced apoptosis, whereas overexpressing aCDase in these cells enhanced their resistance to apoptosis (117). Pharmacological inhibition of aCDase with N-oleoylethanolamine (NOE) and RNAi knockdown of aCDase both sensitize mouse and human hepatoma cells to daunorubicin by increasing ceramide levels and preventing the conversion of ceramide to anti-apoptotic S-1-P within the cells (118). Furthermore, overexpression of aCDase in prostate tumor cells inhibited increases in ceramide levels by doxorubicin and etoposide, thus suppressing cell apoptosis induced by these and other chemotherapeutic agents including cisplatin and gemcitabine (119). These observations suggest the regulation of aCDase may mediate the effect of chemotherapeutic agents by decreasing the levels of ceramide that is released in response to these agents. Such results are indicative of aCDase having a protective role in cell survival. The importance of aCDase in survival is manifested though the embryonic lethality of an aCDase gene knockout in mice (120), whereby the gene product, aCDase, is critical for the removal of ceramide from newly formed embryos.

Contrary to aCDase, alkaline CDases (alkCDases) are the most recent CDases to be identified and cloned. Once thought to be just one enzyme, there are at least three mammalian genes encoding alkCDases: alkCDase-1, alkCDase-2, and alkCDase-3. All three alkCDases have been cloned from both humans and mouse, and each shares a high degree of protein sequence identity between the two species (40, 121, 122, 123, 124, 125). The three alkCDases are homologous to each other with alkCDase-1 and alkCDase-2 having a higher degree of protein similarity between each other (125). The alkCDases range between 264 and 275 AA in length and are rarely, if at all, post-translationally modified. Both human and mouse alkCDase-1 are predominantly
expressed in the skin and localize to the ER within cells (121, 123). Human alkCDase-1 prefers very long-chain unsaturated ceramides (>C20) as substrates and has optimal enzymatic activity around pH 8.5 (121). Human alkCDase 2, however, has a wider pH range (7.4-9.0) of optimal activity and prefers long chain or very-long chain ceramides (40). Human alkCDase localizes to the Golgi apparatus, and although it is still unclear, mouse alkCDase probably localizes to the Golgi also (40, 124). Interestingly, alkCDase-3 (optimal pH 9.5) was actually the first identified alkCDase, which was probably due to the fact that it is highly and ubiquitously expressed (122). Subcellularly, alkCDase-3 localizes to both the ER and Golgi where it likely only uses long-chain (C16 – C20) unsaturated ceramides, including dihydroceramides and phytoceramides, as substrates (122). Unlike the other CDases, the activities of alkCDases are dependent upon Ca\(^{2+}\).

While three types of alkCDase have been identified, little is known about their roles in regulation of cellular responses. As alkCDase-1 localizes mainly within the skin, it is thought to play a role in the differentiation of epidermal keratinocytes. Specifically, RNAi knockdown of alkCDase-1 inhibited Ca\(^{2+}\)-induced growth arrest of human keratinocytes, while concomitantly attenuating the expression of keratin 1 and involucrin, both markers of differentiated keratinocytes (121). AlkCDase-2 is thought to possibly have a cytoprotective effect against serum-induced apoptosis. HeLa cells were shown to upregulate alkCDase-2 in response to serum starvation, allowing for sufficient production of S-1-P and activation of rS1P-1 to promote cell survival and serum-free proliferation (40). No direct role in cellular responses such as proliferation, apoptosis, migration, or differentiation has been determined for alkCDase-3. Although, they are highly and ubiquitously expressed, alkCDases’ endogenous substrates, including unusual long-chain
ceramides, dihydroceramides, and phytoceramides, are found in very low abundance within cells. Therefore it is postulated that this class of CDases may act as housekeeping enzymes to catabolize specific groups of ceramides that are poor substrates for the other CDases (125).

Before being cloned, alkCDases and nCDases were often referred to as one enzyme for a couple reasons. The first was because they have overlapping pH ranges for activity, and secondly, there was little evidence supporting multiple distinct enzymes. This changed at the turn of the century with the cloning of the human nCDase gene. Human nCDase was initially cloned as a 763 AA protein that was shown to localize to mitochondria (126). Subsequent cloning of mouse and rat nCDases revealed that these nCDases localized in the plasma membrane (127, 128). This led to the cloning of the human nCDase gene again, which showed the full length human nCDase was 782 AA long and localized in the plasma membrane (129). Human nCDase is ubiquitously expressed (130) as is mouse nCDase, which has high expression in kidneys, liver and heart, medium expression in the brain and lung, and low levels in the spleen, skeletal muscle, and testes (127). NCDases from human, mouse, and rat are all glycosylated, which is necessary for their localization to the plasma membrane. The substrate specificity for human nCDase is uncertain, however both rat and mouse nCDases prefer long chain ceramides (130, 131). Moreover, mouse nCDase preferentially metabolized C_{16}- and C_{18}-ceramides over C_{12}-ceramides and preferred regular ceramides over dihydroceramides (131). While human nCDase has a broad pH range (7-9) for optimal activity (126), mouse and rat nCDases are both optimally active at a pH of 7.5 (130, 131).
The role of nCDase in regulating biological responses is still relatively unknown as the cloning and identification of nCDase occurred more recently than aCDase, upon which much research had been focused. Knocking out nCDase in mice was not embryonic lethal (132), suggesting, at a minimum, that other CDases could compensate for a lack of nCDase. For the most part, nCDase knockout mice were healthy, had no major abnormalities, and had normal ceramide levels in most tissues. Ceramide levels were only shown to be high in certain areas of the intestine in these knockout mice, which correlated with an increased rate of apoptosis in these areas (132). These findings suggest that nCDase plays an important role in the catabolism of dietary sphingolipids. Furthermore, evidence has accumulated demonstrating the importance of nCDase in regulating ceramide levels in response to cytokine- and growth factor-mediated signaling.

We have previously shown that platelet derived growth factor increased nCDase activity in rat mesangial cells (133). Also, nCDase may have a role in regulating ceramide that is released by various cytokines or chemotherapeutics which generate ceramide through activation of SMases at the plasma membrane. In CHOP (Chinese Hamster Ovarian cells expressing Polyoma LT antigen) cells, nCDase was only involved in altering ceramide, sphingosine, and S-1-P levels when ceramide was released from sphingomyelin in the plasma membrane, but not from when ceramide was generated by de novo synthesis (134). This is not surprising as nCDase is mainly localized to the plasma membrane. Moreover, nCDase was shown to protect against TNF-α-induced ceramide accumulation and apoptosis in primary hepatocytes and also against TNF-α-induced hepatotoxicity in a rat in vivo model (135). The cytoprotective effects of nCDase were also demonstrated to be dependent on the subsequent conversion of CDase-generated sphingosine into S-1-P.
In addition, nCDase activity was regulated by the cytokine interleukin-1β (IL-1β) in a bimodal manner in rat hepatocytes (136). Furthermore, after an initial release of ceramide, long-term exposure of mesangial cells to IL-1β results in cytoprotective up-regulation of nCDase mRNA expression and protein synthesis (137). Degradation of nCDase induced by nitric oxide (NO) results in apoptosis from increased ceramide levels. Activation of nCDase via PKC dependent phosphorylation prevented its degradation and reversed the effects of NO (138). These observations suggest that nCDase may be important in protecting cells against inflammatory agents that generate the release of ceramide from sphingomyelin.

1.6 Gene Transcription

All enzymes, including sphingolipid metabolizing enzymes are proteins, and as such, their synthesis starts with transcription of their respective genes. Gene transcription is the process by which mRNA transcripts are derived from DNA encoding the gene. The generated mRNA transcripts then serve as instructions for ribosomes to combine amino acids (AA) in ordered sequences into peptides, otherwise known as translation. RNA polymerase II is a complex enzyme that synthesizes mRNA from the coding region of DNA, using the 3’—5’ DNA strand as its template. Thus, the transcribed mRNA is 5’—3’, having the same sequence as the coding, or 5’—3’, strand of DNA, except uridine in the RNA substitutes for thymidine in DNA. RNA polymerase II cannot just freely attach to DNA and begin transcription; its recruitment and attachment to DNA is highly regulated to prevent rampant or insufficient gene expression. The binding of RNA polymerase II is mediated by a specific complex of transcription factors. Transcription
factors comprise a plethora of proteins that directly, or indirectly, bind DNA and mediate transcription initiation either positively or negatively. Transcription factors can directly bind to DNA through interacting with a specific DNA sequence known as a transcriptional response element (TRE), or a transcription factor binding site. Alternatively, transcription factors can indirectly bind with DNA by forming a complex with or binding to other transcription factors already bound to DNA. Initiation of transcription by RNA polymerase II requires the binding of five general transcription factors (GTFs) to make the transcription initiation complex: TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (139). A sixth GTF, although not necessary for transcription, greatly enhances transcription by stabilizing the binding of TFIID to the TATA box, a TRE containing the core DNA sequence “TATA.” TFIID contains a TATA-binding protein (TBP) which allows for direct binding of TFIID to DNA. Once TFIID binds to DNA, RNA polymerase II and the remaining GTFs form a complex with TFIID, thus allowing gene transcription to proceed (139). Although the TATA box TRE is found in many genes, it is not required for transcription, and other DNA elements may participate in the initiation of transcription. Genes lacking a TATA box often have at least one CCAAT box and/or at least one GC box that may be involved with the initiation of transcription (139).

Besides the TATA, CCAAT, or GC boxes, a plethora of other TREs are possibly present within DNA 5’-upstream of a gene’s transcriptional start site. A multitude of transcription factors can bind these TREs in response to any number of cellular signals, thus contributing to the transcriptional regulation of the gene. The proximal DNA upstream of a gene’s transcriptional start site, with its multiple TREs, makes up the promoter region. Transcription factors can also bind TREs that are more distal from the
transcription start site, sometimes thousands of base pairs away. Such TREs are often referred to as enhancers or repressors depending on if they, respectively, activate or repress transcription. DNA conformational changes allow enhancers/repressors, despite their distal location, to communicate with RNA Polymerase II within the initiation complex (139). This communication occurs through an 18-subunit complex termed Mediator, which associates with GTFs and RNA Polymerase II to make the complete transcription initiation complex (139). The concerted interactions of transcription factors binding to enhancers, repressors, and promoter TREs effectively regulate transcription by controlling the formation and/or stability of the GTF/RNA polymerase II complex (140).

All transcription factors that recognize TREs in DNA contain two characteristic domains: a DNA binding domain and a trans-activating domain. The DNA binding domain recognizes and binds to specific DNA sequences, i.e., TREs. Once bound to DNA, the activation domain allows a transcription factor to recruit coactivators and/or transcription machinery. In addition, the bound transcription factor could also exert repressing effects on transcription, though the respective TREs for such factors usually are located more distal from the transcription start site. Over 2000 transcription factors exist, thus the combinatorial possibilities of a set of transcription factors that could regulate a gene are so vast that every gene could theoretically be regulated by a unique set of transcription factors (140). Transcription factors can be broadly classified into two categories, constitutively-active or regulatory. Constitutively-active transcription factors always reside within the nucleus with full activation potential where they can regulate genes that are constantly transcribed or work together with regulatory transcription factors in controlling a multitude of other genes (140). Regulatory transcription factors
can either be regulated developmentally or via cellular signals. The genes for developmental transcription factors are turned on in different stages of development in a cell type specific manner, i.e., spatiotemporally regulated (140). Signal-dependent transcription factors remain inactive (minimally active) until they are activated by signal transduction pathways (140). After which, the activated transcription factors can exert their transcription regulatory effects.

For this review, we will limit our discussion of individual transcription factors to those that potentially bind to putative TREs identified within a region of DNA that our studies indicate may be the human nCDase proximal promoter. One of the most abundant signal-dependent activated transcription factors and ubiquitously expressed is AP-1. It is activated via MAPK pathways which phosphorylate AP-1 in response to a plethora of stimulating factors including, but not limited to, cytokines, stress signals, growth factors, and oncogenic stimuli (141). AP-1 is a dimer consisting of subunits from Jun (c-Jun, JunB, JunD, and v-Jun), Fos (c-Fos, v-Fos, FosB, Fra-1, and Fra-2), ATF (activating transcription factor), or JDP (Jun dimerization protein) families (142). Jun family members either form homodimers or heterodimers with the other subunit families to make complete AP-1 complexes. No matter from what family, all AP-1 subunits have a basic DNA binding domain combined with a leucine zipper region that is necessary for dimerization. Once dimerized, the DNA binding domains of each subunit cooperatively interact to bind the AP-1 TRE (143). The DNA consensus sequence for this TRE, or AP-1 cis-element, is 5’-TGA(G/C)TCA-3’, though several sequence variations have been reported to bind at least one of the many possible AP-1 dimers (143). Interestingly, the various subunit family members differ in activation potential from one subunit to the
next. C-Jun, c-Fos, and FosB usually are strong activators, whereas JunB, JunD, Fra-1, and Fra-2 are usually weak activators (142). In fact, AP-1 dimers containing the weak subunits can repress the effects of AP-1 dimers with stronger activation potential (142). AP-1-induced up- and down-regulation of gene expression has a role in multiple biological responses including differentiation, apoptosis, transformation, and proliferation. The role of AP-1 in differentiation is exemplified by the prevention of osteoclast formation and disruption of osteoblastogenesis through the targeted disruption of specific AP-1 dimers (144, 145). Depending on the spatiotemporal conditions, activation of AP-1 can induce apoptosis by upregulating pro-apoptotic genes like FasL (Fas ligand) and Bim; contrarily, activated AP-1 could down regulate a pro-apoptotic gene, e.g., Fas, or up-regulate an anti-apoptotic gene, e.g., Bcl3, to induce an overall anti-apoptotic effect (141). AP-1 can also be transforming. In fact, c-Jun and c-Fos are homologues of retroviral oncogenes (146, 147). AP-1 has been demonstrated to regulate proliferation of many cell types. It has been shown to promote proliferation of liver cells and keratinocytes (148, 149), and also, AP-1 was shown to mediate fibroblast proliferation by regulating cell cycle control via inducing cyclin D1 expression (150, 151). In addition, activation of AP-1 can induce vascular SMC proliferation. In support of this, the therapeutic agent magnolol, which inhibits the development of atherosclerosis, decreased vascular SMC proliferation, in part, by lowering AP-1 activation (152). This indicated that AP-1 has potential roles in both atherosclerosis and restenosis. Further roles of AP-1 in atherosclerosis are demonstrated by increases in vascular SMC migration in response to AP-1-dependent activation of matrix metalloproteinase-9 (153).
Like AP-1, the transcription factor NF-Y is ubiquitously expressed. However, unlike AP-1, NF-Y is a constitutively active transcription factor, which recognizes one of the most ubiquitous promoter elements, i.e., a CCAAT box. The core DNA sequence of a CCAAT box is CCAAT, as its name suggests. A few different transcription factors can bind the CCAAT box, and because of this, the flanking nucleotides can help determine specificity. The binding of NF-Y to the CCAAT box is highly dependent on the flanking nucleotides, and mutating these nucleotides has the potential to severely affect the binding of NF-Y to the CCAAT box (154). NF-Y can bind a CCAAT box in the forward or reverse orientation, and in TATA-less promoters, more often than not, the CCAAT box is found in the reverse orientation (155). NF-Y is a trimeric protein consisting of subunits NF-YA, NF-YB, and NF-YC which are all necessary for DNA binding (156). Interestingly, the NF-Y TRE is invariably flanked by at least one other TRE, whereby NF-Y facilitates the binding and/or activation potential of the transcription factor binding to the other cis-element (157). Because of this, NF-Y can be a necessary regulator of developmentally- or tissue-specific controlled genes (158, 159), housekeeping or inducible genes (160, 161), or cell cycle regulated genes (155), despite itself being a constitutively active transcription factor.

The transcription factor Oct-1 is another ubiquitously expressed transcription factor. It belongs to the octamer family (Oct) of transcription factors, which bind the consensus octamer motif, 5’-ATGC(A/T)AAT-3’, or other related 8 bp sequences (162). Oct transcription factors belong to the POU (Pit Oct Unc) subgroup of homeodomain proteins (163). The Oct transcription factors contain a POU-specific domain connected to a homeodomain forming a helix-turn-helix DNA binding domain (162).
family, only Oct-1 is not expressed in a spatiotemporal pattern. Oct-2 is found in cells of
B-cell lineage and the central nervous system and, also, in some monocytes and
macrophages (164). Oct-3/4 and its strict regulation are absolutely necessary for
embryonic stem (ES) cells to maintain their pluripotency; decreases in Oct-3/4 levels
result in differentiation of ES cells (165). Oct-6 is expressed strongly in certain parts of
the nervous system and is important for development of various cell types, including
Schwann cell differentiation (166). Oct transcription factors have non-redundant
functions as Oct-1 knockout mice are embryonic lethal and die in late gestation (167).
Oct-1 is crucial to the regulation of multiple types of genes. It has been shown to be an
important regulator of essential housekeeping genes such as the histone H2B gene (2).
Oct-1 also helps regulate some stress-induced genes, such as *Sesn2, AMPKɑ2, and Nxn*
that are critical for cells’ adaptations to stress (168). Interestingly, Oct transcription
factors can function as both positive and negative regulators, depending on the gene,
tissue, or stage of development (169, 170).

Similar to the octamer family of transcription factors, the AP-2 (activator protein
2) family consists of several AP-2 transcription factors designated by Greek naming, e.g.,
AP-2ɑ, AP-2β, and AP-2γ. Five different AP-2 proteins have been identified in humans
and mouse, all sharing a highly conserved helix-span-helix motif that is necessary for
dimerization (171). Once dimerized, a highly conserved basic domain, in conjunction
with the helix-span-helix domain, is able to bind the palindromic consensus sequence 5’-
GCCN$_3$GGC-3’ (172). However, the various AP-2 transcription factors have been shown
to bind to several variants of the consensus sequence, indicating that a range of G/C-rich
elements may act as AP-2 binding sites, albeit with different affinities for AP-2 (172).
The N-terminus contains a proline and glutamine rich trans-activating domain which is less conserved among the different AP-2 proteins (173). AP-2 localizes in the nucleus and can be activated through protein kinase A (PKA)-mediated phosphorylation (174). Generally, AP-2 has roles in suppressing terminal differentiation during embryonic development, and in adult cells, it can regulate cell-type-specific proliferation. AP-2α is expressed in primitive ectoderm tissue and is necessary for proper differentiation of neural-crest cells (175, 176). AP-2β has been shown to be necessary for kidney development (177). In addition, AP-2 has been linked to carcinogenesis as AP-2α and AP-2γ levels were found elevated in several types of cancer including testicular, ovarian, and breast cancers (178, 179, 180).

The GATA family of transcription factors is another family of multiple transcription factors that have highly conserved DNA binding domains. GATA transcription factors contain both a C- and an N-terminus zinc-finger of the sequence Cys-X2-Cys-X7-Cys-X2-Cys. The C-terminal zinc finger was shown to be indispensable for GATA DNA binding (181, 182), whereas the N-terminal zinc-finger can bind transcriptional cofactors or adjacent cis-elements (183, 184). Regulation of many genes by GATA factors requires the binding of FOG (friend of GATA) to the N-terminal zinc finger (185). The conserved binding domains of the GATA factors have a strong and specific affinity for the DNA sequence (A/T)GATA(A/G) (186, 187). Conservation in the trans-activation domain allows for the classification of GATA factors into two subfamilies, GATA-1-like (GATA-1, GATA-2, GATA-3) and GATA-4-like (GATA-4, GATA-5, and GATA-6). GATA-1, -2, and -3 are highly expressed in hematopoietic stem cells and have crucial roles in regulating genes necessary for initial proliferation of
hematopoietic precursors and differentiation of erythroid cells, megakaryocytes, and T-lymphocytes (188, 189, 190). GATA-1-like GATA factors are also found in non-hematopoietic cells including cells in gonads, brain, and kidney in both embryonic and differentiated stages (191, 192). GATA-4-like proteins were originally detected as important regulators of both regulatory and structural genes in cardiac tissue, but since then, GATA-4, -5, and -6 were shown to be critical factors in tissue-specific gene regulation in other tissues including the liver, gut, gonads, and lung (193, 194, 195, 196, 197, 198). Interestingly, in VSMCs, GATA-6 was shown to have a role in limiting proliferation by regulating the gene expression of the cell cycle inhibitor p21 (198, 199). In response to the above observations, it has been proposed that the polytropic roles in gene expression that GATA factors have, including their ability to regulate differing tissue-specific expressed genes in multiple tissues, occur through specific interactions of GATA with other transcription factors that are expressed in semi-restricted patterns (182).

1.7 Summary

Atherosclerosis is an inflammatory disease of the arteries and an underlying cause of a vast number of deaths annually. A significant body of work demonstrates that there is a relationship with sphingolipid levels and the atherosclerotic disease state. The sphingolipids ceramide, sphingosine, and sphingosine-1-phosphate have defined roles in biological processes such as proliferation, migration, and apoptosis. New data suggests other sphingolipids also have important biological roles in cellular processes. Most sphingolipids are derived from reversible metabolic processing of ceramide. Because
sequential sphingolipid metabolites can have different or opposing cellular effects, it is of
equal importance to understand the roles and functions of sphingolipid metabolizing
enzymes. Furthermore, our lab has demonstrated the therapeutic potential of using
exogenous ceramide as an inhibitor of stenosis (107). The overall purpose of research
described in this dissertation was to elucidate the therapeutic potential of ceramide in
preventing stenosis/restenosis. Specifically, the effects of ceramide on the vasculature
using in vivo and in vitro models were explored. Differences in ceramide metabolism
between the two main cell types of human arteries were compared to explain possible
differential effects of the cells to ceramide, i.e., decreased vascular SMC growth despite
increases in vascular EC wound healing responses. A crucial enzyme in the generation of
sphingosine, and consequently S-1-P, from ceramide is nCDase. To further elucidate the
role of nCDase in ceramide metabolism, transcriptional regulation of human nCDase was
also investigated.
Chapter 2

Identification and Characterization of Transcriptional Response Elements for Human Neutral Ceramidase
2.1 Abstract

Many forms of cellular stress elevate endogenous ceramide levels, which is implicated in inducing cell growth arrest or apoptosis. Ceramidases (CDase) play a critical role in regulating apoptosis by hydrolyzing ceramide into sphingosine, a precursor for promitogenic sphingosine-1-phosphate. Growth factor activation of neutral CDase (nCDase) has been shown to have a cytoprotective effect against cytokine-induced increases in ceramide levels. To define the transcriptional regulation of nCDase expression, we used a luciferase reporter analysis system to define the proximal promoter as a 200 bp region of the 5’-untranslated region of the nCDase gene. We demonstrated that serum activated this proximal promoter, which correlated with a serum-induced increase in human nCDase mRNA expression. Computational analysis revealed the proximal promoter region contains several well-characterized transcriptional response elements (TREs), lacks a canonical TATA box, but did contain a reverse oriented CCAAT box. Electrophoretic mobility shift assays demonstrated that the identified candidate TREs bind their respective transcription factors, including AP-1, NF-Y, AP-2, Oct-1, GATA. Mutagenic analyses of the TREs revealed that these sites regulate the promoter and are necessary for maximal activity of the proximal promoter. As serum induced promoter activity, we focused on the serum-regulated AP-1 cis-element. SiRNA knockdown of the AP-1 subunit, c-Jun, inhibited the activity of the human nCDase proximal promoter, whereas, c-Jun overexpression increased activity. Taken together, our findings suggest that c-Jun/AP-1 signaling may, in part, regulate serum-induced human nCDase gene transcription.
2.2 Introduction

Mounting evidence has demonstrated that sphingolipid metabolites, including ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S-1-P), are critical modulators of cellular function. Ceramide and Sph are second messengers shown to induce cell growth arrest or apoptosis (200, 201). In contrast, S-1-P promotes cell growth (202) and differentiation (47, 203) and can suppress the apoptotic effects of ceramide (204). Hence, it is postulated that overall balance of intracellular levels of sphingolipid metabolites, in part, determines cellular responses.

Sph, the precursor for S-1-P, is formed solely through the catalytic activity of CDases. CDases cleave the N-acyl linkage of ceramide, yielding Sph and free fatty acids as products (205). CDases are classified into three subtypes according to their pH optima: acidic, neutral, and alkaline. Evidence suggests that nCDases are critical physiological enzymes in regulating the balance of sphingolipid metabolites. Human, rat, and mouse nCDases have all been defined as integral membrane proteins localizing to the plasma membrane (129, 206), where second-messenger ceramide is predominantly generated in response to cytokines and cellular stress. Furthermore, rat kidney nCDase was shown to be enriched in structured membrane microdomains (SMD), also known as lipid rafts (128). Not only has nCDase been shown to localize to SMD, but exogenously delivered ceramide also localized to these domains (207). Also, we have previously shown that ceramide recruits and activates a downstream target, PKCζ, within SMD (208). The abilities of nCDase to modulate lipid mediated signaling in response to various cytokine and growth factor stimuli are supported by the co-localization of enzyme, substrate, and targets within SMD.
The physiological consequences of altering ceramide mass are becoming more apparent. Many forms of cellular stress, including, but not limited to, heat shock, ionizing radiation, ultra violet light, Fas ligand, growth factor removal, and oxidative stress cause an elevation of endogenous ceramide levels (202, 209). Similarly, many anticancer drugs mediate apoptosis by elevating levels of ceramide (210, 211). Evidence has accumulated demonstrating the importance of nCDase in regulating ceramide concentration in response to cytokine- and growth factor-mediated signaling. We have previously shown that platelet derived growth factor increased nCDase activity in rat mesangial cells (133). In addition, nCDase activity was regulated by the cytokine interleukin-1β (IL-1β) in a bimodal manner in rat hepatocytes (136). Furthermore, after an initial release of ceramide, long-term exposure of mesangial cells to IL-1β resulted in cytoprotective up-regulation of nCDase mRNA expression and protein synthesis (137). Degradation of nCDase induced by nitric oxide (NO) results in apoptosis as a result of the increase in ceramide. Activation of nCDase via PKC dependent phosphorylation prevented its degradation and reversed the effects of NO (138). Moreover, nCDase was shown to protect against TNFα-induced ceramide accumulation and apoptosis in primary hepatocytes and also against TNFα-induced hepatotoxicity in a rat in vivo model (135). Despite the increasing evidence of the critical role of nCDase in cellular function, little is known concerning its transcriptional regulation.

Previous studies have identified, but not confirmed, putative transcriptional response elements (TREs) in the mouse nCDase promoter (127). The goal of this present study was to characterize the transcriptional machinery that regulates the human nCDase gene. We have identified a putative proximal promoter of the human nCDase gene that is
serum-inducible and contains multiple TREs, including AP-1, CCAAT, AP-2, Oct, and GATA elements, which are necessary for maximal promoter activity. Identifying the mechanisms underlying transcriptional regulation of nCDase may identify new targets in diseases such as cancer and atherosclerosis where promitogenic sphingolipid metabolites are generated.

2.3 Materials and Methods

2.3.1 Cell Culture

Human embryonic kidney 293 (HEK 293) cells were obtained from American Type Culture Collection (Rockville, MD). Passages 5-20 were maintained in DMEM (GIBCO-Invitrogen) supplemented with 10% fetal bovine serum (FBS). Human coronary artery smooth muscle cells (HCASMC) were obtained from Cascade Biologics, Inc. (Portland, Oregon). Passages 3-10 were maintained in Medium-231(Cascade Biologics, Inc.) supplemented with Smooth Muscle Cell Growth Supplement (Cascade Biologics, Inc.).

2.3.2 Cloning of 5’-Flanking Region of Human NCDase Gene

To obtain the 5’-flanking sequence of the human nCDase gene, human GenomeWalker kit (Clontech, Mountain View, CA), containing four libraries of human genomic DNA, was used according to manufacturer’s instructions. Human nCDase gene specific primers GSwalk1 (5’-TTCAATGGTCCCACCTGGTGAATAAACAAG-3’) and GSwalk2 (5’-AGAAGGGCCACTGTGGTGACTCAT-3’) were designed and
synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primary polymerase chain reaction (PCR) was carried out using 1 uL of genomic DNA, gene specific primer GSwalk1 and adaptor primer AP1, and Advantage 2 Polymerase Mix (Clontech). The genomic library was amplified by PCR (94°C for 2 sec, then 72°C for 3 min for 7 cycles, followed by 32 cycles of 94°C for 2 sec, then 67°C for 3 min). A final extension step was done at 67°C for 4 min. The primary PCR products were diluted 1:50 with sterile water and used as a template for a second PCR reaction with the nested gene specific primer GSwalk2 and the nested adapter primer AP2. The secondary PCR was performed at the same temperatures as the primary reaction with 5 and 20 cycles, respectively. The reaction was again held at 67°C for 4 min for final extension. The final PCR products were separated by agarose gel electrophoresis, cloned into pGEM-T Easy vector (Promega, Madison, WI), and sequenced in both directions using pUC/M13 forward and reverse primers for pGEM-T and internally designed primers.

2.3.3 Sequence Analysis

Putative transcription factor binding sites were predicted with Transcriptional Element Search Software (TESS) (http://cbil.upenn.edu/tess/). Sequencing comparisons between and alignments of human and mouse nCDase putative promoter regions were accomplished using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.3.4 Construction of Reporter Genes

Specific sequences of the 5’-flanking regions of the human nCDase gene were amplified by PCR. The nucleotide range of each amplified region and the respective
5’ forward primers used to amplify them are as follows: -3000/-1 TRE (CPF3-KpnI: 5’-GAGCGGTACCTATCAATACTCTTTAATCTCATAC-3’), -2500/-1 TRE (CPF4-KpnI: 5’-GAGCGGTACCATAATTATGGCTTAGCTCTAC-3’), -2000/-1 TRE (CPF5-KpnI: 5’-GAGCGGTACCCATAATTATGGCTTAGCTCTAC-3’), -1500/-1 TRE (CPF6-KpnI: 5’-GAGCGGTACCAGTGCTCATCATATTTTACGAATT-3’), -1000/-1 TRE (CPF7-KpnI: 5’-GAGCGGTACCATAAGATGATCATAATCATATTCC-3’), -500/-1 TRE (CPF8-KpnI: 5’-AGTCGGTACCAGTCCATGACCACAGAGTACTGTC-3’), -400/-1 TRE (CPF400-KpnI: 5’-AGTCGGTACCAGGCCAGACCCATGTACAGATCCA-3’), -300/-1 TRE (CPF300-KpnI: 5’-AGTCGGTACCTCAGTGCCCAATGCTAAATCATGA-3’), -200/-1 TRE (CPF200-KpnI: 5’-AGTCGGTACCATGGATTGCTTTGTCTGTCCATG-3’), -100/-1 TRE (CPF100-KpnI: 5’-AGTCGGTACCAGATATCTTAACCTCGGTTGGCTT-3’), -50/-1 TRE (CPF50-KpnI: 5’-AGTAAGGTACCTCAGGATTGCTTTGTCTGTCCATG-3’). The 3’ reverse primer used to amplify all regions was CPR1-XhoI (5’-TGACCTCGAGTTCTTCTCAGGTACAGCAGAGTG). The underlined bases are restriction digest sites for KpnI for the 5’ primers and XhoI for the 3’ primer. PCR’s were performed using the pGEM-T vector containing the 5’-flanking region as a template. PfuUltra High Fidelity DNA polymerase (Stratagene, La Jolla, CA) was used according to manufacturer’s instructions. The cycling parameters were 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min/kb, for 30 cycles. The DNA fragments generated were cloned into a TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen), propagated in E. Coli, and followed by plasmid isolation with Qiaprep Spin Mini Kit (Qiagen, Valencia, CA). The plasmids containing the 5’-flanking regions of DNA were digested
with *KpnI* and *XhoI*, and the specific regions were purified by gel electrophoresis and cloned into the firefly luciferase expression vector pGL3-Basic vector (Promega) using the same restriction sites.

### 2.3.5 Construction of Site-Directed Mutational Reporter Plasmids

Site-directed mutagenesis or deletion of the putative transcription factor binding sites was done using Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s protocol. Primers used to alter the binding sites were designed with the help of Stratagene’s web-based primer design software program (http://labtools.stratagene.com/QC). Sense primers used are given in Table 2; antisense primers (not shown) are the reverse complement of the given sense primers. PCR was then performed with the appropriate primers using 25 ng of PGL-200 (pGL3-basic vector with –200 to –1 bp of CDase promoter) as a template. The cycling parameters were 95°C for 30 sec, 55°C for 1 min, and 68°C for 5 min, for 12 cycles. The parental DNA was then digested with 1 uL *Dpn* restriction enzyme for 1 h at 37°C. The remaining mutated vectors from the digested DNA were propagated by transformation of XL1-Blue supercompetent cells (Stratagene).

### 2.3.6 Preparation of Nuclear Extract

Nuclear extracts were prepared using the Pierce Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER) (Pierce/Thermo Fisher Scientific, Rockford, IL) according to manufacture’s instructions with slight modifications. Briefly, HEK 293 cells were grown to 90% confluency in a T175 flask, detached with trypsin, and isolated
by centrifugation to yield a packed cell volume of approximately 300 uL. After the addition of 3 ml of CERI (cytoplasm extraction reagent I) buffer from the NE-PER kit, the pellet was vortexed and incubated on ice for 10 min. CERII (165 uL) from the NE-PER kit was then added followed by vortexing and an additional incubation on ice. The nuclear fraction was then separated by centrifugation and the supernatant (cytoplasmic extract) was pipetted off. The pellet containing the nuclei was then resuspended in 375 uL NER (Nuclear extraction reagent) buffer from the NE-PER kit and incubated on ice for 40 min with periodic vortexing. The suspension was centrifuged a final time and the supernatant (nuclear extract) was removed and dialyzed in 4% glycerol, 10 mM Tris (pH 7.5) buffer for two hours to remove excess salt. The nuclear extract was stored at –80°C, and the protein concentration was measured using the Bio-Rad Protein Assay Kit.

2.3.7 Electrophoretic Mobility Shift Assays (EMSA)

All DNA oligonucleotides were synthesized by IDT (Table 2.1). The Gel Shift Assay System (Promega) was used according to manufacturer’s instructions. Synthetic complementary nucleotides were annealed, end-labeled with \[^{32}\text{P}\]ATP (7000Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) and T4 polynucleotide kinase, and purified with Sephadex G-25 Quick Spin Columns (Roche Diagnostics, Indianapolis, IN).

EMSAs assays were performed with 35-40 fmol of double-stranded end-labeled probes and 12-15 ug of HEK 293 nuclear protein for 20 minutes at room temperature. For competition assays, nuclear protein was pre-incubated with a 50-fold excess (except where noted) of unlabeled double-stranded EMSA oligonucleotides for 10 min before the addition of labeled probe. Sequences of labeled probes are given in Table 1.
buffer without dye was added before the protein/DNA complexes were resolved on
Novex 6% DNA retardation gels (Invitrogen) using 1/2x TBE buffer (Sigma Aldrich, St.
Louis, MO) at 200 V for 20 min. For antibody blocking EMSAs, nuclear protein was
pre-incubated with rabbit or goat polyclonal antibodies against Oct-1 (sc-232x) and AP-
2α (sc-184x) obtained from Santa Cruz (Santa Cruz, CA) for 1 h on ice. After
electrophoresis, gels were dried and autoradiographed.

2.3.8 Transient Transfection and Luciferase Assays

Human embryonic kidney 293 (HEK 293) cells (40-60% confluent) were
transfected with each reporter construct (250 ng) and the Renilla luciferase expression
vector phRL-null (100 ng, Promega) using Lipofectamine 2000 (3:1, uLlipofectamine:ugDNA)
(Invitrogen) in 250 uL Opti-MEM (GIBCO-Invitrogen) in 12 well plates. HEK 293 cells
were grown in 1 ml DMEM supplemented with 10% FBS, and the media was not
changed after the addition of the transfection reagents. Twenty-four hours after
transfection, cells were lysed by the addition of 250 uL of Passive Lysis Buffer
(Promega). For serum induced promoter activity, 30-40% confluent HEK 293 cells were
transfected overnight and serum deprived with basal media the next morning for 24
hours. FBS was then added to a final concentration of 10% v/v, followed by lysing at the
time points given in the results section. The luciferase activity in the cell lysates was
determined using the Dual Luciferase Reporter System (Promega). Firefly luciferase
activities of the human nCDase promoter and luciferase gene chimeras were normalized
to that of Renilla luciferase (or to total protein where noted) and expressed relative to the
activity of the pGL3-Basic plasmid. For overexpression of c-Jun and c-Fos, 500 ng of
each vector was co-transfected with the reporter vectors, while maintaining a 3:1 ratio (uL:ug) of Lipofectamine 2000:DNA. For c-Jun knockdown, 10 pmol of c-Jun siRNA (JUN Stealth™ RNAi DuoPak, Invitrogen) was delivered with the reporter DNA in 3 uL of Lipofectamine 2000 per well of a 12-well plate.

2.3.9 Quantitative Real-Time RT-PCR

HCASMC and HEK 293 cells were seeded onto 60 mm tissue culture dishes and six well plates, respectively, and grown to 70-85% (HCASMC) or 60-70% (HEK 293) confluency. Cells were then serum starved in their respective basal media for 24 hours. Subsequently, cells were treated with either vehicle or 10% FBS for the time points given prior to RNA extraction. RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Inc, Valencia, CA) according to manufacturer’s protocol. RNA quantity and quality were assessed using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano Assay (Agilent, Palo Alto, CA). First strand complementary DNA (cDNA) was transcribed from 1 µg RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer’s protocol. The relative expression of human nCDase was quantified by quantitative real-time polymerase chain reaction (QRT-PCR) assay using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), maintained at Penn State College of Medicine Functional Genomics Core Facilities. Taqman gene expression assays containing both primers and probes for each gene were purchased from Applied Biosystems.

Quantitative PCR was carried out on a real-time detection instrument (ABI 7900HT Sequence Detection System) in 384-well optical plates using Taqman Universal PCR Master Mix and Assay on Demand primers and probes (Applied Biosystems)
similar to previously described methods (212, 213). Reactions were carried out in 10 µL volume, containing 2X TaqMan Universal PCR Master Mix with UNG, 25 ng cDNA from reverse transcribed RNA, 450 nM unlabeled Taqman gene specific primers, and 125 nM FAM dye-labeled Taqman MGB probe. The reaction was held at 50ºC for 2 min and then held at 95ºC for 10 min before undergoing 40 cycles of a denaturation step at 95ºC for 15 sec and an annealing/extension step at 60ºC for 1 min. Relative quantities were calculated using ABI SDS 2.2.2 RQ software (Applied Biosystems, Inc.) and the $2^{\Delta\Delta Ct}$ analysis method(214) with GAPDH as the endogenous control. Final results are given as relative expression normalized to vehicle/serum-starved samples.

2.3.10 Statistical Analysis

The results are expressed as mean ± standard error of the mean of at least three independent experiments. Probability (p) values ≤ 0.05 (Student’s t-test) were considered to indicate statistically significant differences.

2.4 Results

2.4.1 Serum induces human nCDase mRNA expression through proximal regulatory sequences

As ceramide metabolism has been linked to the formation of promitogenic phosphorylated metabolites, we investigated if serum treatment induced nCDase expression. We initially chose human coronary artery smooth muscle cells (HCASMC) as a model system, since we had previously reported that balloon angioplasty induced
smooth muscle cell migration and proliferation, which correlated with increased
promitogenic signaling cascades and metabolism of ceramide (107, 215). Treatment with
fetal bovine serum (FBS) induced approximately a 60% increase in nCDase mRNA
expression in HCASMC after 10 hours of treatment (Fig. 2.1A). This increase in nCDase
expression was seen as early as 6 hours, and remained significantly elevated 16 hours
after treatment. In the next series of experiments, we investigated serum-regulated
human nCDase in HEK 293 cells, a more transfection-friendly cell line. FBS elevated
nCDase mRNA expression approximately 35% in HEK 293 cells (Fig. 2.1B). Significant
increases in nCDase expression occurred four hours after treatment and persisted through
12 hours. Taken together, these data suggested that serum components might
transcriptionally regulate the expression of nCDase.
Figure 2.1 Serum induces human nCDase mRNA expression in HCASMC and HEK 293 cells. Log phase growing cells were serum deprived in basal media for 24 h and then treated with serum (10% final concentration) for the indicated number of hours. Following serum treatment, total RNA was extracted and used as the initial template in quantitative real-time RT-PCR analysis. Relative quantities were calculated using the $2^{\Delta\Delta Ct}$ analysis method (214) with GAPDH as the endogenous control. Final results are shown as relative expression normalized to serum starved samples treated for 0 hours with serum. A) Relative nCDase expression in HCASMC in response to serum was measured over a 16 hr time period. B) Relative nCDase expression in HEK 293 cells in response to serum was measured over a 12 hr time period. Values are expressed as mean ± S.E.M. Asterisk indicates significance, $p \leq 0.05$, relative to 0 hrs treatment, from at least 3 individual experiments performed in triplicate.
In order to better understand the molecular mechanisms by which serum induces transcription of human nCDase, we examined the promoter region of the human nCDase gene. To identify the human nCDase promoter region, the DNA 5’ upstream of the open reading frame (ORF) of the nCDase gene was obtained using a PCR-based genomic walking method. Using the GenomeWalker™ Kit containing human genomic DNA along with CDase specific primers, a 3.0 kb fragment of the 5’-flanking region of the human nCDase gene corresponding to the nucleotides –3000 to –1, relative to the translation start site, was cloned into a general cloning vector (pGEM-T easy). After aligning sequences with ClustalW software, there was only 46% nucleotide sequence identity between the mouse nCDase promoter (216) and the putative human nCDase promoter as compared to 81% cDNA sequence identity for the ORF between the two species (217). Hence, transcriptional regulation of nCDase appears to be less evolutionarily conserved than the enzyme itself, thus it is likely that the nCDase transcriptional response elements may be different in humans compared to murine or other species.

In order to determine whether the unique initial 3000 bp of the 5’-UTR of human nCDase had promoter activity, we cloned this 5’-region into the 5’-end of pGL3-basic luciferase reporter vector to create PGL-3000. The luciferase activity from the HEK 293 cells transfected with PGL-3000 was about 15-fold higher than that from cells transfected with the pGL3-basic control vector (Fig. 2.2A, PGL-3000 vs. PGL-Basic), indicating that the 3000 bp region functions as a promoter for the human nCDase gene. To localize DNA elements responsible for this transcriptional activity, successive 500 bp deletions of the 5’-flanking region were generated by site-directed PCR amplification and inserted into
**Figure 2.2** Deletion analysis of the 5'-UTR identified a 200 bp region as a serum-inducible, proximal promoter of the human nCDase gene. Firefly luciferase (fLuc) expression vectors containing sequentially smaller regions of the nCDase promoter were cotransfected into HEK 293 cells with a plasmid (pRL-null) containing the *renilla* luciferase (rLuc) gene. After 24 h incubation, post-transfection, with DMEM containing FBS, fLuc and rLuc activities were measured. The fLuc/rLuc ratios were determined, and means (±S.E.M) for a minimum of three transfections were calculated. Values are expressed as fold increase relative to the luminescence ratio observed in pGL3-Basic transfected cells (relative value = 1). A) Sequential 500 bp deletion analysis of the cloned 3000 base pair nCDase promoter region was used to identify regions likely to contain activator and/or repressor regions. Single asterisk represents significance compared to PGL-Basic. Double asterisks represent significance compared to PGL-3000. B) Sequential deletion analysis of bp –500 to –1 of the 5’-UTR identified the proximal promoter as the region containing the major positive regulatory elements of the human nCDase gene. Single asterisk represents significance compared to all data sets. C) Luciferase activity of the human nCDase proximal promoter is increased through serum treatment. HEK 293 cells, transfected overnight with PGL-200, were serum starved for 24 hours and then treated with serum or basal media for the indicated time points. Values are expressed as mean ± S.E.M-fold increase in luminescence relative to serum starved PGL-200 transfected cells. Single asterisk represents significance compared to serum starved samples.
the pGL3-basic vector to construct PGL-2500, PGL-2000, PGL-1500, PGL-1000, and PGL-500. These vectors were then transiently transfected into HEK 293 cells and assayed for luciferase activity. Luciferase activity of PGL-Basic was set as negative control with a value of 1, and all other activities normalized to the control vector. Luciferase activity was highest from the reporter construct carrying a fragment from –500 to –1 (Fig. 2.2A, PGL-500). Successive increases in 5’-flanking fragment length resulted in a continued decrease in luciferase activity through 2500 bp, where the activity from PGL-2500 was decreased by >80% compared to PGL-500 (Fig. 2.2A). These results suggest that positive regulatory elements may exist between bp –500 and –1 and also upstream of bp –2500, whereas negative regulatory elements are likely located between bp –2500 and –500. Most notably, the luciferase activity from the reporter construct containing the shortest fragment (PGL-500) was even higher than the activity from the full-length construct.

In order to further delineate where the promoter regulatory elements lie within this 500 bp region, successive deletions of the sequence were inserted into pGL3-basic to make PGL-400, PGL-300, PGL-200, PGL-100, and PGL-50. Luciferase activity was highest from the reporter construct carrying a fragment from –200 to –1 (Fig. 2.2B). In fact, activity from PGL-200 showed over 40-fold increased luciferase activity compared to the control pGL-basic vector, whereas the PGL-500 showed an approximate 20-fold increase. Deletion of bp –200 to –51 of the 200 bp 5’-flanking region completely abolished all luciferase activity (Fig. 2.2B). These results indicated that the major positive regulatory elements of the human nCDase gene reside within this 200 bp region.
of the 5’-flanking sequence, establishing this sequence as the proximal promoter region of the human nCDase gene.

We then determined whether if this proximal promoter region could be transcriptionally activated by FBS. For this, HEK 293 cells were transiently transfected with PGL-200 and then serum deprived for 24 h. The transfected cells were then treated with 10% FBS and luciferase activity was measured at various time points. After 16 hours, FBS increased luciferase activity approximately 25% compared to serum starved cells (Fig. 2.2C). Furthermore, FBS induced luciferase activity was significantly elevated 36 h post treatment. This correlated well with the induction of human nCDase mRNA by FBS (Fig. 2.1A & 2.1B). Small changes in lipids can have very significant effects on cellular signaling. Therefore, regulation of enzymes that alter lipid species must be very tightly controlled. A serum-induced 25% increase in nCDase promoter activity has the potential to lead to biologically significant changes in sphingosine or S-1-P levels through upregulation of nCDase expression.

2.4.2 Identification of transcriptional response elements within the 200 bp human nCDase proximal promoter

The above data indicate that the 200 bp proximal promoter region contains TREs, which could possibly bind to serum-stimulated transcription factors. To identify the TREs that may be responsible for nCDase transcriptional activity, the 200 bp region of human nCDase gene 5’-UTR was analyzed using Transcription Element Search Software (TESS). This software is designed to search nucleotide sequences for potential transcription factor binding sites using site or consensus strings and positional weight
matrices from TRANSFAC 6.0. TESS analysis indicated that the proximal promoter contained multiple potential transcription factor binding sites. These include putative AP-1, AP-2, SP-1, Oct, RSRFC4 (related to serum response factor C4), and GATA sites, and also a CCAAT box (Fig. 2.3). Interestingly, the analyses did not detect a TATA box throughout the entire 5’-UTR of the human nCDase gene.

2.4.3 The 200 bp human nCDase proximal promoter contains a functional overlapping AP-1/CCAAT binding site.

Given that the 200 bp proximal promoter contained multiple putative transcription factor binding sites, EMSAs were performed to validate the functional role of these binding sites. We first examined the overlapping putative AP-1 and CCAAT box cis-
elements for two reasons. First, serum and growth factors are known to activate the transcription factor AP-1. Second, CCAAT boxes have been shown to have important roles in regulating TATA-less genes (155, 157). To determine if the putative AP-1/CCAAT site of the human nCDase gene can functionally bind transcription factors, EMSAs were performed using nuclear extracts prepared from HEK 293 cells and a $^{32}$P-radiolabeled 22-nucleotide probe (bp –204 to –183) containing the putative AP-1/CCAAT site centrally positioned, designated “Ap1/ccaat” (see Table 1 for sequence). The results from EMSAs revealed two distinct shifted bands (Fig. 2.4A, lane 2) compared to the control labeled probe without nuclear extract (Fig. 2.4A, lane 1). Preincubation with 50-molar excess of unlabeled Ap-1/ccaat oligonucleotide completely inhibited the binding of nuclear proteins to the labeled probe (Fig. 2.4A, lane 3), indicating the specific interaction of potential transcription factors with AP-1/CCAAT DNA. To rule out non-specific DNA-protein interactions, pre-incubation with 50-molar excess of an unlabeled, nonspecific oligonucleotide (bp –108 to –87 of the proximal promoter) did not affect the shifted bands (Fig. 2.4A, lane 4). These results indicated the putative AP-1/CCAAT binding site binds specifically to nuclear proteins from HEK 293 cells.
Figure 2.4 Competition EMSAs revealed the proximal promoter of the human nCDase gene contains overlapping Ap-1 and NF-Y binding sites with different nucleotide specificity for binding. Nuclear extracts from HEK 293 cells were subjected to EMSAs with radiolabeled Ap1/ccaat probe (Table 1) containing nucleotides –204 to –183, with radiolabeled AP-1 probe (Table 1), or radiolabeled NFY probe (Table 1). Lanes containing each labeled probe are underlined with the corresponding labeled probe marked below the underlined lanes. Oligonucleotides used as competitors are marked above the corresponding lanes. Unlabeled Ap1/ccaat, AP-1 consensus, C/EBP consensus, NF-Y consensus, and non-specific oligonucleotides were used as competitors in a 50-fold excess in the corresponding lanes. A) EMSAs demonstrated that unlabeled AP-1 consensus oligonucleotide is a competitor against binding of nuclear extract proteins to labeled probe containing the putative Ap1/CCAAT binding site (probe Ap1/ccaat). B) Conversely, EMSAs also demonstrated that unlabeled Ap1/ccaat oligonucleotide competitively inhibits binding of labeled AP-1 consensus probe to nuclear extract proteins. C) EMSAs demonstrated that unlabeled NF-Y consensus oligonucleotide is a competitor against binding of nuclear extract proteins to labeled Ap1/ccaat probe. D) Conversely, EMSAs also demonstrated that unlabeled Ap1/ccaat oligonucleotide competitively inhibits binding of labeled NF-Y consensus probe to nuclear extract proteins. E) Alignment of wild-type sequence (WT) of region –204/-183 found in the human nCDase promoter and oligonucleotides used as competitors (labeled Mutant A-D) is shown. The introduced mutations in oligonucleotides Mutant A-D are underlined. These unlabeled oligonucleotides were used as additional competitors in part F, where the WT oligonucleotide was used as the labeled probe. The putative AP-1 binding site is underlined with a solid line in the WT sequence, whereas the putative NF-Y site is over scored with a dashed line. F) Nuclear extracts were subjected to EMSAs with radiolabeled WT (Ap1/ccaat) probe as described above. In addition, mutated oligonucleotides were used as competitors in 50-fold excess in the corresponding lanes.
In order to determine whether AP-1 is the transcription factor that directly binds to AP-1/CCAAT DNA, competition EMSAs were performed using an unlabeled 21 bp oligonucleotide containing the AP-1 consensus binding sequence as a competitor (Table 2.1, AP1). As shown in Figure 2.4A, an excess of unlabeled AP1 oligonucleotide completely inhibited the lower band while not affecting the upper band (Fig. 2.4A, lane 5). This suggested that the lower band was the result of the binding of AP-1 to the Ap1/ccaat probe. In order to further confirm that the lower band is indeed the AP-1 transcription factor binding to the Ap-1/ccaat probe, additional EMSAs were performed using $^{32}$P-labeled AP1 consensus oligonucleotide as a probe. Labeled AP1 probe formed a complex that migrated with the lower band of labeled Ap1/ccaat (Fig. 2.4A, lane 6 vs. lane 2). More importantly, a 50-molar excess of unlabeled Ap-1/ccaat oligonucleotide inhibited formation of the labeled AP-1 band (Fig. 2.4B, lane 4). The fact that the Ap1/ccaat oligonucleotide is inhibited by AP1 consensus oligonucleotide indicates that the putative AP-1/CCAAT sequence in the human nCDase is able to bind with AP-1 transcription factors.
<table>
<thead>
<tr>
<th>TF/Ligand</th>
<th>Sense Oligonucleotide (5'-3')</th>
<th>Antisense Oligonucleotide (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ap1/ecaat</td>
<td><strong>CTGTAAT</strong>GATTTGGTCTTTTCTC**</td>
<td><strong>AGACAAGAC</strong>CAATCA<strong>ATTACAG</strong></td>
</tr>
<tr>
<td>Ap2/Sp1</td>
<td><strong>TTGCTG</strong>CCATGGGCTTTGGCTATGG</td>
<td><strong>CCATAG</strong>ACCAAGCCATGGCA<strong>GACAA</strong></td>
</tr>
<tr>
<td>Rsrfc4/Oct</td>
<td><strong>ATAGTC</strong>TATTAATGAAAGAGAAG</td>
<td><strong>CTCTTT</strong>CATTATTAGACTAT</td>
</tr>
<tr>
<td>Gata</td>
<td><strong>AAGAGCTA</strong>AGATATCTTAACCTTAC</td>
<td><strong>AAGAGCTA</strong>AGATATCTTAACC</td>
</tr>
<tr>
<td>Oct-b</td>
<td><strong>TTAAAGC</strong>ATTTGCAATTAAATT</td>
<td><strong>AATCAG</strong>ATGCAAA<strong>GCTTTAA</strong></td>
</tr>
<tr>
<td>AP1</td>
<td><strong>CGCTTGATGAGTCAGCCGGAGA</strong></td>
<td><strong>TTCCGGCTGACTCATCAAGCG</strong></td>
</tr>
<tr>
<td>NFY</td>
<td><strong>AGCCGTACGATGATTGGTTAATCT</strong></td>
<td><strong>AGAGGATTACAATCAGTC</strong></td>
</tr>
<tr>
<td>AP2</td>
<td><strong>GATCGA</strong>ACTGACCCTCGGCTTCATC</td>
<td><strong>ACGGGCCCTCGGGCGGCTC</strong></td>
</tr>
<tr>
<td>SPI</td>
<td><strong>ATTCGAT</strong>CGGCGGCGGCGGCGG</td>
<td><strong>GCTC</strong>GGCCGC<strong>CCGGCTCGAAT</strong></td>
</tr>
<tr>
<td>GATA</td>
<td><strong>CAGT</strong>TGATACGTTTCTGGTACCTG</td>
<td><strong>AGAG</strong>TTACATTTCTCTT</td>
</tr>
<tr>
<td>OCT-1</td>
<td><strong>TGTC</strong>GAATGCAAATCAGGAA</td>
<td><strong>GCTA</strong>GAATTGCA<strong>ACTGAG</strong></td>
</tr>
<tr>
<td>CEBP</td>
<td><strong>TGCAGG</strong>ATTGC<strong>GCAATCT</strong>GC</td>
<td><strong>TGCAGA</strong>TTGCG<strong>CAATCT</strong>GCA</td>
</tr>
</tbody>
</table>

Bold letters in oligonucleotide sequences indicate putative TF binding sites.
Although we identified the lower band as an AP-1/DNA complex, the identity of the trans-activator(s) responsible for forming the upper band was still unknown. We hypothesized that transcription factors binding to the overlapping CCAAT box would generate the upper band. Several transcription factors have been shown to bind the DNA sequence CCAAT, including CCAAT/Enhancer Binding Protein (C/EBP) and Nuclear Factor Y (NF-Y) (157). To determine the transcription factors comprising the upper protein/probe complex, competition EMSAs were performed. We utilized oligonucleotides containing either NF-Y or C/EBP consensus-binding sequences (“NFY,” “CEBP,” Table 1) as competitors to the labeled Ap1/ccaat probe. Both bands were unaffected by preincubation with a 50-molar excess of unlabeled CEBP (Fig. 2.4C, lane 5). In contrast, an excess of unlabeled NFY oligonucleotide was able to significantly inhibit the formation of the upper band while minimally affecting the lower band (Fig. 2.4C, lane 6). This suggested that the upper band was the result of binding of NF-Y to the Ap1/ccaat probe. As a control for specific binding, EMSAs were performed using a reverse strategy whereby the oligonucleotide containing the NF-Y consensus sequence was used as a labeled probe. Labeled NFY consensus probe generated a band (Fig. 2.4D, lane 1) that was inhibited using unlabeled NFY oligonucleotide (Fig. 2.4D, lane 2), but was unaffected by unlabeled non-specific oligonucleotides (Fig. 2.4D, lanes 3 & 4). Most importantly, excess unlabeled Ap1/ccaat oligonucleotide drastically reduced the intensity of the band formed by binding of NF-Y protein to labeled NFY consensus probe (Fig. 2.4D, lanes 5 & 6). These results indicate that the transcription factor NF-Y binds to the AP-1/CCAAT sequence of the human nCDase gene.
2.4.4 The AP-1 binding site overlaps, but is not dependent on, a functional NF-Y binding CCAAT box.

We next utilized mutant oligonucleotide competitors to decipher the binding of distinct *trans*-activating factors to the functional overlapping AP-1/CCAAT box. These studies were designed to determine which nucleotides are necessary for AP-1 and/or NF-Y binding. We performed competition EMSAs with excess unlabeled mutant oligonucleotides and labeled Ap1/ccaat probe. One or two base-pair mutations (mutants A-D) were introduced into the Ap-1/ccaat oligonucleotide (Fig. 2.4E) to determine the necessity of those nucleotides for binding AP-1 or NF-Y. The unlabeled wild type probe (lane 4) effectively competed for binding to both complexes, while the unlabeled AP1 consensus probe competed for only the lower AP-1 complex band (lane 3). The putative AP-1 binding sequence is –197 T G A T T G G –191, while the putative CCAAT-binding factor sequence is –196 G A T T G G –191. Mutation of nucleotide –191 from a G to a T (Mutant A) competed with the labeled wild type probe for binding with AP-1, but not NF-Y (Fig. 2.4E, lane 5). Simultaneous mutation of nucleotides –190 and –189 from T and C to G and A, respectively, also prevented NF-Y binding but did not inhibit AP-1 binding (data not shown), thus demonstrating that specific nucleotides directly adjacent to the CCAAT box are necessary for binding of NF-Y (155, 157). In contrast, mutating position –195 from an A to G (mutant B) dramatically decreased the oligonucleotide’s affinity for AP-1, but not NF-Y. A mutation at nucleotide –197 from T to C (mutant C) slightly decreased the affinity of the oligonucleotide for both AP-1 and NF-Y (Fig. 2.4F, lane 7). Mutant D contained two mutations (nucleotides –197 T to C and –196 G to C), prevented binding of the oligonucleotide to both AP-1 and NF-Y (Fig. 2.4F, lane 8).
These results further support the binding of both AP-1 and NF-Y to an overlapping AP-1/CCAAT binding site. Furthermore, these results indicated that, despite an almost complete overlap of binding site nucleotides, the nucleotides necessary for binding of each transcription factor are partially independent.

### 2.4.5 C-Jun, but not c-Fos, regulates the AP-1 transcriptional site on human nCDase proximal promoter.

As we identified a functional AP-1 TRE in the proximal promoter of human nCDase, we next investigated the role of serum-responsive AP-1 transcription factors in the regulation of the proximal promoter. We initially chose to investigate c-Jun as it is the most common subunit of AP-1 transcription factors. We co-transfected dual siRNA’s (Invitrogen) directed against c-Jun with PGL-200 into proliferating HEK 293 cells and assayed for luciferase activity. Ten pmol of c-Jun siRNA successfully knocked down c-Jun protein levels 40-60% (Fig. 2.5A). Knocking down c-Jun expression using c-Jun siRNA reduced the luciferase activity by 20% compared to cells co-transfected with scrambled siRNA and PGL-200 (Fig. 2.5B). This result demonstrated that a functional AP-1 protein, consisting of at least one c-Jun subunit, is necessary for maximal human nCDase promoter activity.
Figure 2.5 AP-1 is necessary for maximal human nCDase proximal promoter activity and sufficient to induce its activity.  

A) Knockdown of c-Jun with siRNA indicated that AP-1 is necessary for maximal activity of the 200 bp human nCDase promoter. HEK 293 cells were co-transfected with siRNA against c-Jun or scrambled siRNA and the PGL-200 luciferase reporter in DMEM containing FBS. fLuc activity was measured 24 h post-transfection and normalized to rLuc activity. Cell lysates were analyzed by western blots to determine c-Jun knockdown efficiency.  

A) Representative autoradiograph from Western analyses demonstrates the knockdown of c-Jun.  

B) The c-Jun siRNA-induced decrease in luciferase activity is expressed relative to the activity from scrambled siRNA-treated cells, where an asterisk marks a significantly different decrease in activity compared to scrambled siRNA treated cells. Results represent the mean ± S.E.M of at least three individual experiments.  

C) Overexpression of c-Jun indicated that AP-1 is sufficient to increase the activity of the human nCDase proximal promoter. HEK 293 cells were co-transfected with the PGL-200 luciferase reporter vector and either c-Jun expression vector, c-Fos expression vector, both c-Jun and c-Fos vectors, or empty vector. fLuc activity was measured 24 h post-transfection and normalized to rLuc activity. Reporter activity is expressed relative to the luciferase activity from cells co-transfected with the empty vector (Mock). Results represent the mean ± S.E.M of at least three individual experiments. Significant changes in relative expression are marked with an asterisk.
The role of AP-1 to induce the proximal promoter from the human nCDase gene was further tested using c-Jun and c-Fos expression vectors, named oeJun and oeFos, co-transfected with PGL-200 into proliferating HEK 293 cells. The transcription factor AP-1 consists of Jun-family/Jun-family dimers or Jun-family/Fos-family dimers. Overexpression of c-Jun induced a 34% increase in luciferase activity relative to the activity of cells transfected with PGL-200 alone (Fig. 2.5C). Overexpression of c-Fos had no significant effect on luciferase activity. Co-transfection of both oeJun and oeFos with PGL-200 induced an approximate 45% increase in luciferase activity compared to PGL-200 alone (Fig. 2.5C), which was not significantly different from the activation with c-Jun alone. These data suggested that AP-1 transcription factors could induce the human nCDase promoter. Specifically, AP-1 with c-Jun subunit(s) was sufficient to mediate this induction, whereas a c-Fos subunit appeared to have little functional importance.

2.4.6 The human nCDase proximal promoter contains two functional Octamer binding sites.

In addition to the AP-1/CCAAT site, TESS analysis identified a putative binding site for another serum-regulated transcription factor, RSRFC4 (related to serum response factor-C4). We examined if the putative RSRFC4 site was able to function as an actual trans-activator binding site for nCDase. TESS analysis also revealed that the RSRFC4 site overlaps with an Oct (Octamer-binding) site, starting at nucleotide –118 (Fig. 2.3). We designed and labeled a double-stranded, 22 bp oligonucleotide probe, from bp –125 to –104 (Table 2.1, Rsrfc4/Oct), containing the putative sites to identify any transcription
factors that may bind this overlapping TRE. The $^{32}$P-labeled Rsrfc4/Oct probe showed a band shift following incubation with the nuclear extract from HEK 293 cells (Fig. 2.6A, lane 2). Competition EMSAs with excess amounts of the identified unlabeled oligonucleotides (Fig. 2.6A, lanes 3&4) indicated that the lower band in Figure 2.6 represents specific interactions between nuclear proteins and the Oct/Rsrfc4 probe and that the apparent upper band is the result of non-specific DNA-protein interactions.
Figure 2.6 Competition EMSAs revealed the human nCDase proximal promoter contains two binding sites with varying affinity for Oct-1 at positions −118/-110 and −71/-64. Nuclear extracts from HEK 293 cells were subjected to EMSAs with radiolabeled Rsrfc4/Oct probe (Table 1), Oct-b probe (Table 1), or with radiolabeled OCT-1 probe (Table 1) containing the Oct-1 consensus sequence. Lanes containing each labeled probe are underlined with the corresponding labeled probe marked below the underlined lanes. Unlabeled Rsrfc4/Oct, Oct-b, OCT-1 consensus, or non-specific oligonucleotides were used as competitors in a 50-fold excess to determine the specificity for Oct-1 binding.

Oligonucleotides used as competitors are marked above the corresponding lanes. A) The putative Oct-1 site between nucleotides −118/-110 bound to Oct-1, but with a weaker affinity than the OCT-1 consensus oligonucleotide. B) The putative Oct-1 site between nucleotides −71/-64 bound to Oct-1 with comparable affinity as the Oct-1 consensus oligonucleotide. C) EMSAs with blocking antibodies confirmed the binding of Oct-1 to the putative Oct-1 cis-element at position −71/-64. Additional EMSAs were performed using the radiolabeled Oct-b probe with the addition of antibodies to induce supershifts. Antibodies against Oct-1, antibodies against AP-2α (for control), and the amounts of antibodies used in each blocking assay are marked above their corresponding lane. Positive antibody blocking assays resulted in decreased labeled probe/protein complex (band) intensity.
To identify which nuclear protein(s) specifically bound the putative RSRFC4/Oct site, competitive EMSAs were performed with Oct-1 consensus oligonucleotide (Table 2.1, OCT1). An excess of unlabeled OCT1 almost completely prevented a band shift with labeled Oct/Rsrfc4 probe (Fig. 2.6A, lane 5). Furthermore, 32P-labeling of the OCT1 probe revealed a specific DNA/Oct-1 complex induced band shift correlating with the aforementioned lower band (Fig. 2.6A, lane 7 vs. lane 2). This band was significantly attenuated by competition with a 50-molar excess of unlabeled Oct/Rsrfc4 oligonucleotide (Fig. 2.6A, lane 10). These results indicate that the putative Oct/RSRFC4 site in the human nCDase proximal promoter is a functional Oct binding site.

A second putative Oct site was also identified in the human nCDase proximal promoter starting at nucleotide -71 (Fig. 2.3). A 32P-labeled 22 nucleotide (bp –78 to –57) probe containing this binding site centrally positioned (Oct-b, Table 2.1) was used in EMSAs to determine its functionality. These EMSAs resulted in a distinct, specific DNA/transcription factor band shift (Fig. 2.6B, lanes 2-4). Competition assays with unlabeled OCT1 consensus oligonucleotide and labeled OCT1 probe confirmed the second putative Oct site bound to an Oct transcription factor (Fig. 2.6B, lane 5-10). To determine that the transcription factor binding the Oct-b probe is Oct-1, EMSAs with blocking antibodies were performed. Inclusion of an antibody against Oct-1 in the incubation stage of EMSAs continuously decreased the intensity of the shifted band with increasing concentrations of antibody (Fig. 2.6C, lanes 4-6). This decrease was specific to the anti-OCT-1 antibody, since increasing concentrations of an antibody directed against a different transcription factor did not affect the shifted band (Fig. 2.6, lanes 7 and 8). The results from Figure 2.6B and the blocking antibody (Fig. 2.6C) clearly showed
that Oct-1 transcription factor bound to the second putative Oct site within the human nCDase proximal promoter.

2.4.7 The human nCDase proximal promoter contains both functional AP-2 and GATA binding sites

The proximal promoter of the human nCDase gene also contains a putative SP-1 binding site partially overlapping an AP-2 binding site (Fig. 2.3), which was of interest because cooperation between NF-Y and SP-1 in regulating promoters has been demonstrated for multiple genes (155), and SP-1 cis-elements have been found to be functionally important in many TATA-less promoters (218). EMSAs using a $^{32}$P-labeled 26-nucleotide oligonucleotide (bp –188 to –163) containing the putative SP-1 and AP-2 sites centrally positioned (Table 2.1, Ap2/Sp1) resulted in one major band shift, indicating specific binding between transcription factors and the labeled probe (Figure 2.7A, lanes 1-4).
Figure 2.7 Competition EMSAs reveal the human nCDase proximal promoter contains binding sites for AP-2 and GATA factors. Nuclear extracts from HEK 293 cells were subjected to competition EMSAs with the indicated radiolabeled probes. Lanes containing each labeled probe are underlined with the corresponding labeled probe marked below the underlined lanes. Oligonucleotides used as competitors are marked above the corresponding lanes. A) EMSAs with the Ap2/Sp1 probe (Table 1) containing the putative AP-2 site at nucleotides –182 to –175 or with radiolabeled AP2 probe (Table 1) containing the AP-2 consensus sequence. To rule out SP-1 binding, an unlabeled SP-1 oligonucleotide (Table 1) containing the SP-1 consensus sequence was also used, in addition to the AP-2 consensus sequence, as a competitor against labeled Ap2/Sp1 probe. B) EMSAs with the Gata probe (Table 1) containing the putative GATA site at nucleotides –100 to –93 or with radiolabeled GATA probe (Table 1) containing the GATA binding factor consensus sequence.
Since the Ap2/Sp1 probe formed only one major complex, while containing two putative binding sites, it was next determined which transcription factor, AP-2 or SP-1, preferentially interacts with the DNA. Competitive EMSAs with oligonucleotides containing either AP-2 consensus sequence (Table 2.1, AP2) or SP-1 consensus sequence (Table 2.1, SP1) demonstrated that AP-2, but not SP-1, likely bound to the Ap2/Sp1 probe (Fig. 2.7A, lane 5 vs. 6). Furthermore, when AP2 was $^{32}$P-labeled and used in EMSAs, it formed a shifted band that aligned with the band in lane 2 (Fig. 2.7A, lane 7) and that was inhibited by unlabeled Ap2/Sp1 oligonucleotide (Fig. 2.7A, lane 10). Taken together, these results indicated that the putative AP-2/SP-1 sequence in the human nCDase proximal promoter is able to bind the transcription factor AP-2, but not SP-1, in HEK 293 cells.

TESS analysis also revealed a possible GATA-factor binding site (Fig. 2.3). EMSAs using a 22 bp (bp –108 to –87), $^{32}$P-labeled probe containing this putative GATA site centrally positioned (Table 1, Gata) demonstrated there was specific binding of transcription factors to this oligonucleotide (Fig. 2.7B, lanes 1-4). Competition assays with unlabeled GATA consensus oligonucleotide suggested the putative GATA site bound to a GATA transcription factor (Fig. 2.7B, lanes 5). Furthermore, when GATA oligonucleotide was $^{32}$P-labeled and used in EMSAs, a shifted band that aligned with the band in lane 2 (Fig. 2.7B, lane 7) was observed and was inhibited by unlabeled Gata oligonucleotide (Fig. 2.7A, lane 10). Taken together, these results indicated that the putative GATA sequence in the human nCDase proximal promoter is able to bind a transcription factor from the GATA family.
2.4.8 Multiple cis-elements are functionally important for human nCDase proximal promoter activity

Several transcription factors that bind to cis-elements within the human nCDase proximal promoter have been identified. To explore the biological relevance of each of these transcription factor-binding sites, the sites were individually disrupted by introducing mutations into the proximal promoter/reporter vector as shown in Table 2.2. Mutation of the AP-2, GATA, Oct-a (bp –118 to –110), and Oct-b (–71 to –64) cis-elements down regulated luciferase activity 41%, 44%, 49%, and 28%, respectively, compared to the luciferase activity of HEK 293 cells transfected with PGL-200 (Fig. 2.8). To address the importance of the AP-1/CCAAT element to the overall activity of the proximal promoter, we introduced a 9 bp deletion into the PGL-200 reporter vector, eliminating the entire element (ΔAP-1/CCAAT). This deletion significantly, albeit less dramatically, reduced luciferase activity by 14% compared with the wild-type PGL-200 reporter (Fig. 2.8). The data correlated well to the level of reduction in promoter activity observed when c-Jun was knocked down with c-Jun siRNA (Fig. 2.5A). As disruption of these individual binding elements was not sufficient to abrogate all luciferase activity, it implies that multiple and/or combinatorial transcription factor regulation is necessary for maximal activity of the human nCDase proximal promoter. These data further suggest that the activity of the proximal promoter depends on concerted interactions of multiple transcription factors.
Table 2.2

<table>
<thead>
<tr>
<th>TF site</th>
<th>Original sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer sequence (sense strand)&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1 /CCAAT</td>
<td>TGATTGGTC</td>
<td>5’-cagaaccttctctcactgggtcctgaat-3’</td>
</tr>
<tr>
<td>Ap-2</td>
<td>GCCATGGG</td>
<td>5’-ctatcgataagtttacatatggggtttgtctATTGATGctgttctgtaggtgaggtgaggtgaggg-3’</td>
</tr>
<tr>
<td>Rsrf4/ Oct</td>
<td>TATTAATATG</td>
<td>5’-aagtgctctgtaagtttacatatggggtttgtctATTGATGctgttctgtaggtgaggtgaggtgaggg-3’</td>
</tr>
<tr>
<td>Gata</td>
<td>AGATATCT</td>
<td>5’-ggtatagtttactataatagcagcactAGCTTAATGTTTATCTTACATGATGCTTTTGTTGTTTAAGCATTGTAAGGTTG-3’</td>
</tr>
<tr>
<td>Oct-b</td>
<td>ATTTGCAT</td>
<td>5’-gatatcagcctgtgggtttagggtaagGCCTACGTtaatatatgttctctctctcagt-3’</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotides underlined were altered.
<sup>b</sup> Binding site shown in caps. Altered nucleotides are shown in bold.
<sup>c</sup> Deleted nucleotides are represented by a dash, ‘-’
<sup>d</sup> Antisense primers are the reverse complement of respective sense primers
The identified cis-elements are each individually required for maximal activity of the human nCDase proximal promoter.

Mutations within the respective putative transcription factor binding sites (see Table 2) were introduced into the PGL-200 vector, and transfections were performed using HEK 293 cells. After 24 h incubation with DMEM containing FBS, firefly luciferase activity was measured and normalized to renilla luciferase activity. A) The binding sites for AP-2, GATA, and Oct-1 were each individually mutated to prevent binding. The putative AP-1/NF-Y site was deleted from the PGL-200 vector. The results are represented as fold increase in activity from cells transfected with the pGL-3 basic control vector. Significantly different decreases in activity in the mutated vectors compared to PGL-200 are marked by an asterisk. Results represent the mean ± S.E.M of at least three individual experiments.
2.5 Discussion

The present study characterized the transcriptional regulation of human nCDase. Serum was able to induce human nCDase transcription, and promoter reporter assays identified the human nCDase proximal promoter, which was also activated by serum. EMSA analyses demonstrated that putative \textit{cis}-elements were able to bind several transcription factors, such as AP-1, NF-Y, AP-2, Oct, and GATA. Although each transcription factor binding site contributed to promoter activity, basal activity of the proximal promoter was not dependent on any individual transcription factor. These facts further raise questions about the complexity of the interplay of the multiple \textit{trans}-activating factors in regulating human nCDase transcription. It is probable that several factors function in concert with each other to tightly regulate the promoter in response to multiple biological stimuli, including serum. The possible combinations of transcription factors that are necessary to activate the proximal promoter are numerous. Therefore, extensive research will be needed to delineate the role of each \textit{trans}-factor in regulating the human nCDase proximal promoter.

In our study, we focused on the interplay between overlapping TREs in the human nCDase proximal promoter. As an example, we found that the AP-1 binding site overlaps, but is not dependent on, a functional NF-Y binding CCAAT box. To interpret this observation, it is of interest that the putative AP-1 binding site in the human nCDase gene promoter differs from the consensus AP-1 binding sequence, TGA(C/G)TCA. The nCDase AP-1 TRE sequence does, however, match exactly to one of the known AP-1 binding site variants, TGATTGG(219). The first three nucleotides form the first half of the AP-1 consensus sequence, whereas the latter two thirds of the site actually form a
CCAAT box site in an inverted orientation, i.e., 5’-ATTGG-3,’ on the coding strand. In fact, ATTGG is the predominant orientation when it functions as an NF-Y binding element in TATA-less promoters (155). Interestingly, the CCAAT box is invariantly flanked by at least one functionally important *cis*-element (157), which supports the concurrent binding of NF-Y along with AP-1 to the identified AP-1/CCAAT site in the human nCDase proximal promoter. Recently, a different overlapping NF-Y and AP-1 binding site, TATTGGTCAT was discovered in the follicle-stimulating hormone beta (FSHβ) subunit promoter (220). In this site, the NF-Y binding reverse CCAAT box, ATTGG, preceded, yet partially coincided with, the latter half of the AP-1 consensus sequence, GTCA. Interestingly, in the FSHβ promoter, the binding of AP-1 to the novel AP-1 site was contingent upon an intact CCAAT box (220), whereas a mutation of the CCAAT box in the human nCDase promoter had no effect on AP-1 binding, yet prevented the binding of NF-Y (Fig. 2.4E). Similarly, other mutations of the nCDase AP-1 cis-element could disrupt AP-1 binding, without affecting NF-Y binding (Fig. 2.4E).

The mutual interactions between multiple *trans*-activating factors (including AP-1) and NF-Y have been shown to be essential for optimal regulation of transcription (157). NF-Y has the capacity to enhance transactivation through direct protein-protein interactions and/or facilitate the positioning of transcription factors through DNA conformation changes. Although spatial constraints must exist in the binding of two transcription factors to directly adjacent binding sites, our current work and the work by Coss et. al., clearly show that such constraints do not prohibit concurrent binding of AP-1 and NF-Y. Structural and binding characteristics of AP-1 and NF-Y allow hypothetical
circumvention of the spatial constraints. The subunits of an AP-1 dimer form a flexible fork, which could yield to other transcription factors bound in close proximity (220, 221). Subunits of AP-1 bind their corresponding cis-element at the major groove, with only four amino acids in contact with the DNA, while vast portions of the rest of the subunits lie perpendicular to the DNA (221), thus possibly leaving enough space along the DNA for binding of other transactivators (220). Coincidently, NF-Y has been shown to bind to the minor groove of DNA (222), opposite that of AP-1, further minimizing the spatial constraints of AP-1 and NF-Y binding adjacent to each other (220).

Despite having the strongest promoter activity of the nCDase 5’-flanking region and containing a binding site for the potentially serum-activated AP-1, the proximal promoter was only mildly induced by serum, suggesting strict regulation of the nCDase gene. Direct interaction of NF-Y and AP-1 at the AP-1/CCAAT element could help regulate the extent to which serum-activated AP-1 induced transcription. It is also possible that AP-1 and/or NF-Y could interact with other proximate transcription factors. Several other cis-elements in close proximity to the AP-1/NF-Y site were shown to bind their respective transcription factors, such as Oct and GATA factors, which could presumably interact synergistically or opposingly with NF-Y or AP-1. As examples, OCT-1 represses the c-Jun-dependent activation of rat 3alpha-hydroxysteroid oxidoreductase gene promoter (223) or rat CYP4A2 gene promoter (224); yet Oct-1 and c-Jun act synergistically with each other in the regulation of human thromboxane A2 receptor (225), H3.3B histone (226), IL-2, and IL-5 (227) gene promoters. As another example, Oct-1 and NF-Y cooperatively induce the cell cycle-arresting protein Gadd45
promoter (228); while Oct transcription factors limit NF-Y binding and repress HLA-DRA (a cell surface antigen binding protein subunit) transcription (229).

We chose to focus on the transcriptional factors that regulate the overlapping AP-1/CCAAT element. The AP-1 binding site was of interest because the activation of AP-1 by growth factors and serum and its role in proliferation and transformation is well documented (230). AP-1 trans-activators are homo- or heterodimer transcription factors comprising members of the Jun family (c-Jun, JunB, and JunD) and the Fos family (c-Fos, FosB, Fra-1, and Fra-2), with c-Jun being the most abundant AP-1 subunit (230). Our studies demonstrated that overexpressing c-Jun increased reporter activity, revealing that AP-1 is sufficient to induce the human nCDase proximal promoter. In contrast, we observed that overexpression of c-Fos had no effect on promoter reporter activity. However, these results are not surprising when one considers the fact that c-Fos does not form homodimers and, therefore, cannot bind DNA without a Jun-family subunit since dimer formation is a prerequisite to DNA binding. In agreement with this, co-transfection of AP-1 dependent reporters with a c-Fos expression vector resulted in no significant transcription of the reporters in cells lacking c-Jun (231). These studies suggested that c-Jun/AP-1 signaling may be responsible, in part, for growth factor-induced transcriptional regulation of human nCDase (133).

Similar to observations of the murine nCDase gene (216), we found that the 5’-flanking region of the human nCDase gene lacks a TATA box, an important sequence in the initiation of transcription, found in many, if not the majority, of eukaryotic genes. Although it lacked a TATA box, we observed a CCAAT box, in an inverted orientation, within the human nCDase promoter. CCAAT boxes have also been shown to associate
with proteins within the transcription initiation complex (232). Interestingly, the lack of a TATA box (233) and the presence of an in inverted CCAAT box (155) within a promoter are typical features of tightly regulated genes. Thus, it is not surprising that we observe tight transcriptional control of mRNA expression with serum in both HCASMC and HEK 293 cells. Furthermore, signal transduction through sphingolipid second messengers is very sensitive to sphingolipid balance, so minute changes in sphingolipid species can have dramatic cellular effects, which behoove the tight regulation of sphingolipid metabolizing enzymes. Therefore, tight transcriptional control of nCDase mRNA maintains homeostatic control over highly bioactive sphingolipid metabolites.

In this work, we demonstrated that the activity of the human nCDase proximal promoter depends on the coordinated interactions of multiple transcription factors, such as AP-1, NF-Y, AP-2, Oct, and GATA, which are each individually required for maximal activity of the human nCDase proximal promoter. Correlating with the fact that some of these trans-activating factors are serum activated, serum regulated both proximal promoter activity and nCDase mRNA expression. In particular, we demonstrated that c-Jun/AP-1 signaling might, in part, regulate human nCDase gene transcription. Our studies indicate a mechanism by which growth factor stimulated c-Jun/Ap-1 signaling may generate promitogenic sphingosine metabolites at the expense of growth arresting ceramide. These studies have clinical implications in diseases such as cancer and atherosclerosis, where ceramide metabolites contribute to pro-survival and pro-mitogenic phenotypes.

After obtaining a basic understanding of nCDase transcriptional regulation as discussed in this chapter, we next wanted to investigate nCDase in an in vivo model.
Specifically, we were interested in nCDase regulation in the vasculature because initial studies with smooth muscle cell pericytes indicated that ceramide potentially had anti-stenotic properties (234), which could possibly be altered by nCDase because of its role in converting ceramide to S-1-P. We hypothesize that differences in nCDase expression in vascular cells can alter the responses of vascular cells to ceramide. In addition to nCDase, we are also interested in ceramide metabolism as a whole in such an in vivo model and how metabolism affects the efficacy of a sphingolipid-based mimetic drug.
Chapter 3

C₆-Ceramide-Coated Catheters Promote Re-endothelialization of Stretch-Injured Arteries
3.1 Abstract

Drug eluting stents have recently been associated with the increased risk of adverse thrombogenic events and/or late luminal loss, which is highly associated with incomplete re-endothelialization. The increased risks behoove the design of alternative delivery modalities and/or drugs that do not compromise the re-endothelialization process. The objective of the present study is to elucidate the biological mechanism(s) by which non-stent-based delivery modalities for the anti-proliferative lipid metabolite, C6-ceramide, could lead to a reduction in arterial injury after angioplasty. Immunohistochemical studies in rabbit and porcine models suggest that C6-ceramide-coated balloon catheters limit arterial stenosis without inhibiting endothelial wound healing responses. Specifically, C6-ceramide-coated balloon catheters reduce internal elastica injury with a corresponding reduction in medial fracture length in a 28-day porcine coronary artery stretch model. In addition, C6-ceramide decreases the formation of the fibrin matrix to possibly augment the subsequent wound healing response. We hypothesized that differential metabolism of exogenous ceramide by coronary endothelial and smooth muscle cells could explain the apparent discrepancy between the anti-proliferative actions of ceramide and the pro-wound healing responses of ceramide. Human coronary artery endothelial cells (HCAEC), in contrast to human coronary artery smooth muscle cells (HCASMC), preferentially express ceramide kinase and form ceramide-1-phosphate, which promotes endothelial cell survival. The capabilities of HCASMCs and HCAECs to differentially metabolize ceramide offers a mechanism by which ceramide preferentially limits smooth muscle cell growth, in the presence of active wound healing. The combinatorial ability of ceramide to limit vascular smooth muscle proliferation and promote re-endothelialization, offers the potential for C6-ceramide-coated catheters to serve as adjuncts to stent-based modalities or as a stand-alone treatment.
3.2 Introduction

Despite the overwhelming successes with drug-eluting stents, treatment of stenotic injury in patients with complex, tortuous, or diffuse lesions, especially in diabetic patients, is still limited (235, 236). Moreover, drug-eluting stents have recently fallen back into controversy due to the risk of increased thrombotic events and/or late luminal loss (237, 238). In addition, the potentially problematic use of long term clopidogrel treatment in certain populations as well as the cost of stent-based therapies have led to re-evaluation of drug eluting stents (239). As the most useful predictor of thrombotic events with drug-eluting stents is incomplete re-endothelialization (30), therapies that maintain active wound healing while limiting neointimal hyperplasia may be a potentially useful therapeutic strategy. To this end, local delivery via a balloon catheter of a lipophilic drug that limits intimal hyperplasia while promoting endothelial coverage of the balloon-induced injury is an attractive option. Utilizing non-stent-based drug delivery modalities, such as a drug-coated percutaneous transluminal coronary angioplasty balloon catheter, may offer an alternative treatment option for patients with diffuse injury or in-stent restenosis. In fact, a paclitaxel-coated therapeutic balloon has been shown to be a novel method for prevention of restenosis in a porcine overstretch stent model (240), as well as for treatment of human coronary in-stent restenosis (241).

We previously demonstrated that cell-permeable ceramide, which inhibits growth factor-mediated signaling cascades, reduced neointimal hyperplasia without systemic complications in a rabbit carotid model of stretch injury (107). Despite demonstration of the vascular anti-proliferative actions of ceramide-coated balloons, the effects on other
components of restenosis, including re-endothelialization, remodeling, and inflammation, have not been demonstrated. The present study suggests that C6-ceramide-coated balloons limit arterial stenosis without inhibiting endothelial wound healing responses, further support the use of ceramide-coated therapeutic balloons for the treatment of diffuse vascular stenosis as seen in diabetic patients. Moreover, the use of ceramide-coated therapeutic balloons has the potential to promote active endothelial wound healing not only in coronary arteries, but also, in larger diameter arteries.

3.3 Materials and Methods

All animal studies were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.3.1 Porcine Angioplasty Surgery

Mixed breed domestic heparinized (100 mg/kg) male swine weighing between 45 – 56 kg were used in the studies. Access to the arterial system was made through a femoral cut-down and balloon catheters were guided and deployed under fluoroscopy. Appropriate sizing of selected arterials was calculated from x-ray film. 3.3, 5, and 8 Fr balloon dilation catheters were used and sized in the coronary (left anterior descending or circumflex), renal, and iliac arteries, respectively. Sham-operated arteries were used in all experiments. Balloon catheters were inflated three times to manufacturer’s specification (8-10 atm {atmosphere}) for 30 sec intervals. One month after
angioplasty, swine were assessed radiologically and then euthanized. Arteries were
removed, fixed, and processed for hematoxylin/eosin staining. Experiments were
performed at LyChron, Inc. (Mountain View, CA), as well as Penn State College of
Medicine. Similar results were obtained at both facilities. Histochemical analyses for the
experiments run at LyChron were contracted to the Armed Forces Institute of Pathology
(Washington, DC).

3.3.2 Cholesterol Fed Rabbit Angioplasty Surgery

We now extend our previous work in normal-fed rabbits [9], performing
quantitative morphometric analyses of carotid artery intimal hyperplasia and medial
hypertrophy in angioplastied arteries from 2% cholesterol fed New Zealand white (NZW)
rabbits (RSI, Mocksville, NC). The left carotid artery was used as a sham-treated control
in all studies. There were no significant differences between sham-control, vehicle-
control, or ceramide-treated arteries in terms of either tissue wet weight or cellular
protein content. Other controls included ligation of the external carotid artery without
balloon angioplasty, which did not develop neointimal or thrombolytic events in the
common carotid.

3.3.3 Catheter Coating Preparation

3, 5, and 8 Fr balloon dilation catheters were coated with lipid gels (C6-ceramide
(0.5% C6-ceramide [Avanti Polar Lipids, Alabaster, Alabama] in 90:10 ethanol: DMSO)
or dihydro-C6-ceramide (0.5% dihydro-C6-ceramide [BIOMOL International, Plymouth
Meeting, PA) in 90:10 ethanol: DMSO) or vehicle (90:10 ethanol: DMSO)), in double blinded protocol.

### 3.3.4 Histochemistry and Immunohistochemistry

Hematoxylin and eosin staining was performed according to our previously published work (107). For Sudan IV staining, fixed sections were immersed in a 1% Sudan IV in a 1:1 70% ethanol: acetone solution for 5 min. Immunohistochemical staining for PDGF-ββ staining utilized a goat polyclonal antibody for PDGF-ββ (R&D Systems, Minneapolis, MN) and streptavidin horseradish peroxidase (Amersham, Chicago, IL) for visualization.

### 3.3.5 Proliferation and Apoptosis Assays

$^{3}$H-thymidine incorporation (108) and caspase 3/7 activities (207) were used as surrogate markers to assess proliferation and apoptosis, respectively, in human coronary artery smooth muscle cells (HCASMC) (Cascade Biologics, Portland, Oregon) and human coronary artery endothelial cells (HCAEC) (Cell Applications, Inc, San Diego, California). Ceramide metabolites were delivered in 80-90 nm diameter liposomal vesicles as described (207). Control liposomes were made up without ceramide metabolites, but contained the same amount of total lipids.

### 3.3.6 Fibrinogen Adsorption Assay

Poly(DTE carbonate), a well-characterized tyrosine-derived polymer(242), was mixed with paclitaxel or C$_6$-ceramide in a 90:10 ratio using tetrahydrofuran (THF) as a
solvent. The spin-coating of the polymer mix on gold-coated quartz crystals and the
detection of polymer-adsorbed fibrinogen were performed using a Quartz Crystal
Microbalance with Dissipation monitoring (QCM-D) as previously described (243).
Human fibrinogen at a concentration of 3 mg/mL was used for all experiments.

3.3.7 Quantitative Real-time RT-PCR

Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen, Inc, Valencia,
CA) according to manufacturer’s protocol. RNA quantity and quality were assessed
using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano Assay (Agilent, Palo Alto,
CA). cDNA synthesis was performed on total RNA using Superscript III Reverse
Transcriptase (Invitrogen, Carlsbad, CA) and quantitative PCR on samples using
previously described methods (244). Detection used the 7900HT Sequence Detection
System (Applied Biosystems), 384-well optical plates, and Assay-On-Demand (Applied
Biosystems, Foster City, CA) gene specific primers and probes (212), maintained at
Penn State College of Medicine Functional Genomics Core Facilities. The relative
quantities of neutral and alkaline CDase, sphingosine kinase-1, ceramide kinase,
galactosylceramide synthase, and glucosylceramide synthase mRNA expression were
calculated using ABI SDS 2.2.2 RQ software and the $2^{-\Delta\Delta Ct}$ analysis method (214) with
$\beta$-actin as the endogenous control. Final results are given as relative expression
normalized to vehicle treated HCAEC samples.
3.3.8 Lipid Quantification by Mass Spectroscopy

Sphingolipids from HCASMC and HCAEC cells were analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) based on the method described by Merrill et al. (245) and modified by us (246). The mass spectrometry data was collected using an ABI 4000 Q Trap (Applied Biosystems, Foster City, CA) mass spectrometer equipped with a turbo ion spray source. The peak areas for the different sphingolipid subspecies were compared with that of the internal standards.

3.3.9 Toxicology and Toxicokinetic Studies

Toxicology studies were performed via contract services at Redford Laboratories, AR using 6-10 week Sprague Dawley rats or 6-month Beagle dogs. The test article was a formulation of 1.5 mg/mL C6-ceramide in 2% ethanol, 3% PVP, 8% Chromophor EL and 87% water. Dosing solutions were prepared as specified by Pharmatek Laboratories, San Diego or AAI International, Charleston SC; containing GMP grade C6-ceramide obtained from Avanti Polar Lipids (Alabaster AL). In the multiple dose dog studies, the only adverse finding observed was a dose-dependent recovery period in both ceramide and vehicle control groups (prostration, increased saliva, reddened skin), which resolved rapidly, suggesting the transient adverse effects of the high dose ethanol vehicle, not related to the test article. Necropsy, standard histology, clinical chemistry, and coagulation findings were normal.

3.3.10 Statistical Analysis

The results are expressed as mean ± standard error of at least three independent
experiments. Probability (p) values ≤ 0.05 (Student’s t-test) were considered to indicate statistically significant differences. Results of the quantitative real-time RT-PCR were compared using one-way ANOVA.

3.4 Results

3.4.1 C₆-Ceramide-Therapeutic Balloons Reduce Internal Elastica Injury with a Corresponding Reduction in Medial Fracture Length.

C₆-ceramide’s putative role in the prevention of injury-induced stenosis has been based on its well-documented ability to induce cell cycle arrest in VSM cells (108, 247) and to limit neointimal hyperplasia in a balloon injury rabbit carotid artery model (107). We have extended these previously reported data by investigating C₆-ceramide therapeutic balloons in a one-month porcine model of stretch injury. Initially, we evaluated coronary artery injury and resultant stenosis in a 1.3-1 overstretch porcine coronary artery mode. As shown in Fig. 3.1A, C₆-ceramide therapeutic balloons reduced internal elastica injury with a corresponding reduction in fracture sites and in medial fracture length. Moreover, stenotic injury at the fracture sites was significantly reduced. In these n = 6-9 separate, double-blinded, randomized porcine experiments, all of the C₆-ceramide-treated coronary vessels responded with less proliferation and less injury as compared to the corresponding vehicle balloon control arteries. Both medial fracture length and stenosis were reduced by over 50% with ceramide treatment (Fig. 3.1B). As controls, pre- and post-artery diameter, injury ratio, and device diameter per compliance values did not change as a function of ceramide treatment (Fig. 3.1C). These data
support the contention that local acute delivery of C₆-ceramide to injured arteries limits smooth muscle proliferation as a possible consequence of diminishing proliferative signaling cascades (107), diminishing arterial trauma/injury, or promoting wound healing (re-endothelialization) responses.

We next confirmed these porcine coronary experiments with additional studies investigating the effects of C₆-therapeutic balloons on other porcine arterial beds. In Fig. 3.1D, we show that C₆-ceramide therapeutic balloons limit stenosis by greater than 50% in renal and iliac vessels. Again, in these n = 5 porcine two-week experiments, coronary vessel stenosis was also reduced by acute, direct C₆-treatment. Histochemical (Fig. 3.1E) and radiological (Fig. 3.1F) assessments show significant stenosis and occlusion in the control iliac tree after angioplasty that was limited by C₆-ceramide treatment.
Figure 3.1  C6-Ceramide-therapeutic balloons reduce stenosis in multiple arterial beds in both porcine and rabbit models of stretch injury.  A) C6-Ceramide-therapeutic balloons reduced internal elastica injury with a corresponding reduction in medial fracture length in porcine coronary vessels. Two representative photomicrographs from surgeries performed at Lychron, Inc. B/C) Quantification of percent stenosis and percent medial fracture of n = 6-9 pigs (B) and quantification of pre- and post-angioplasty diameters of experimental animals (C). D) C6-ceramide-therapeutic balloons limited stenosis in porcine iliac, renal, and coronary arteries. Quantification of percent stenosis, n = 5 pigs. Surgeries performed at Penn State College of Medicine. E/F) Representative photomicrograph of H & E stained sections (E) and radiological image (F) of porcine iliac arteries treated with C6-ceramide-therapeutic balloons. G) C6-ceramide-therapeutic balloons promote re-endothelialization of rabbit carotid arteries. Increased endothelial cell PDGF-ββ expression was observed three days after treatment with C6- but not vehicle-treated balloons. Representative photomicrograph of n = 3 separate experiments. Multiple authors contributed to this work. Please see Preface for detailed acknowledgment.
3.4.2 C₆-Ceramide-Coated Balloon Limits Arterial Stenosis Without Inhibiting Endothelial Wound-Healing Responses

It appears that overstretch with the C₆-therapeutic balloon resulted in re-endothelialization of the traumatized artery (Fig. 3.1E). To confirm augmented re-endothelialization of damaged, over-stretched arteries with C₆-ceramide in vivo, we utilized the rabbit carotid artery angioplasty model. Three days after angioplasty, we observed that endothelial cells express elevated levels of PDGF-ββ, a hallmark of a healing endothelial lining, after treatment with ceramide-treated, but not control DMSO-treated, balloon catheters (Fig. 3.1G). These data support a role for ceramide-enhanced wound healing to limit stenotic injury.

We utilized an in vitro approach to investigate the mechanism(s) of diminished arterial stenosis, despite active wound healing, after angioplasty with C₆-therapeutic balloons. We directly compared the effects of C₆-ceramide upon HCASMC and HCAEC. As controls, other anti-proliferative therapeutic agents for the vasculature such as rapamycin and paclitaxel were investigated. The ED₅₀ value for growth inhibition of HCASMC by a 24 hr treatment of C₆-ceramide was approximately 100 nmol/L (Fig. 3.2A). Thus, this concentration of ceramide was utilized in further experimentation. 100 nmol/L C₆-ceramide significantly inhibited HCASMC growth (Fig. 3.2B), but not HCAEC growth (Fig. 3.2C), after a 24 hr treatment. As controls, paclitaxel, at an equivalent 100 nmol/L concentration, inhibited both HCASMC and HCAEC proliferation, while rapamycin inhibited HCAEC, but not HCASMC proliferation.

Our lab had previously demonstrated the C₆-ceramide was able to inhibit VSM cell growth through PKCζ-mediated inhibition of AKT (108). In our original studies, we
demonstrated that intercalation of ceramide into rabbit VSM cells correlated with inhibition of stretch injury-associated activation of AKT and, also, extracellular signal-regulated kinase (107). Interestingly, in addition to the exogenously administered C6-ceramide, there may be changes in endogenous ceramide levels and metabolism in response to injury. We did not explore changes in ceramide levels or ceramide metabolism caused by arterial stretch injury. Presently, there is a lack of published data on any effects that arterial stretch injury may have on sphingolipid metabolism. However, it is possible that stretch injury could alter ceramide/sphingolipid metabolism as ceramide metabolism is often activated in response to cellular stress or injury. For instance, activation of acid sphingomyelinase in ischemic cardiac myocytes leads to ceramide production and contributes to ischemia-induced cell death (248). If indeed, stretch injury induces ceramide release, this release could contribute to the hyperproliferation of VSM cells, possibly through shunting of ceramide to pro-mitogenic sphingolipids such as S-1-P. Such proliferative effects of ceramide generation were also observed in VSM cells in response to oxLDL exposure (249). Although at first glance, such results appear to contradict the use of C6-ceramide to inhibit stenosis, these differential responses actually support ceramide’s ability to mediate multiple responses depending on its subcellular localization and eventual downstream metabolism (34). Endogenous ceramide may have a higher propensity to be metabolized into pro-mitogenic sphingolipids compared to synthetic C6-ceramide, which is not a good substrate for many sphingolipid metabolizing enzymes. Regardless of stretch-injury-induced changes in endogenous ceramide, C6-ceramide inhibits VSM cell growth and, subsequently, stenosis after arterial stretch injury.
Figure 3.2. C6-ceramide preferentially decreases human coronary artery smooth muscle cell (HCASMC) proliferation but not human coronary artery endothelial cell (HCAEC) proliferation. A) C6-ceramide decreases HCASMC proliferation as a function of dose. In n = 3 experiments, C6-ceramide, at a concentration of 100 nmol/L, decreases DNA synthesis by approximately 50% in HCASMC as determined via 3H-Thymidine incorporation. B) C6-ceramide, at its EC_{50} concentration, decreases HCASMC proliferation more than paclitaxel and rapamycin at equivalent concentrations, n = 3. C) Paclitaxel and rapamycin, but not C6-ceramide, limit HCAEC proliferation. Thymidine incorporation into HCAEC acid-insoluble DNA is less for paclitaxel and rapamycin, as compared to 100 nmol/L C6-ceramide, n = 3. Multiple authors contributed to this work. Please see Preface for detailed acknowledgment.
3.4.3 HCAEC and HCASMC Differentially Express mRNA for Ceramide Metabolizing Enzymes

The mechanistic explanation for the differential effect of exogenous C₆-ceramide upon vascular smooth muscle and endothelial cells may involve ceramide metabolism. Specifically, we hypothesize that HCAEC preferentially metabolizes exogenous ceramide into ceramide-1-phosphate (C-1-P), a pro-mitogenic, anti-apoptotic second messenger (Fig. 3.3A) (250). Using quantitative real-time polymerase chain reaction analysis (QRT-PCR) to measure gene expression of ceramide metabolic enzymes, we now provide evidence for ceramide metabolism in the diametric responses of vascular endothelial and smooth muscle cells to C₆-ceramide treatment. HCASMC cultures had significantly lower levels of ceramide kinase (the enzyme that forms C-1-P) mRNA relative to levels of ceramide kinase mRNA in HCAEC cell cultures (Fig. 3.3B). The relative levels of ceramide kinase in HCAEC cultures were over twice that of HCASMC cultures. Therefore, HCAEC may have the enhanced capacity to metabolize ceramide into ceramide-1-phosphate (C-1-P). Interestingly, mRNA levels of CDases (neutral and alkaline) and sphingosine kinase, the enzymes responsible for the formation of pro-mitogenic sphingosine-1-phosphate (S-1-P), did not significantly differ between the HCAEC and HCASMC cultures (data not shown).
Figure 3.3 Human coronary artery endothelial cells have an increased capacity to metabolize ceramide into phosphorylated, but not glycosylated, metabolites. (A) Primary ceramide metabolic pathways. The dynamic flux of ceramide metabolites regulates the switch between apoptosis/growth arrest and proliferation. Anti-apoptotic/proliferative phosphorylated metabolites are formed via ceramide kinase or sphingosine kinase. The galactosyl- and glucosylceramide synthases (GCS) metabolize ceramide into less bioactive glycosylated ceramide species. (B-D) Gene expression of sphingolipid metabolic enzymes ceramide kinase (B), galactosylceramide synthase (C), and glucosylceramide synthase (D), in human coronary artery endothelial cells (HCAEC) and smooth muscle cells (HCASMC) was measured by real time RT-PCR. Enzyme mRNA expression levels are all relative to their respective level in vehicle treated HCAECs, (mean ± SEM, n ≥ 5); * Denotes significance compared to vehicle treated HCAECs.
In addition, QRT-PCR analyses demonstrated that HCASMC cultures expressed significantly higher mRNA levels of galactosylceramide synthase and glucosylceramide synthase, relative to levels expressed in HCAEC cultures (Fig. 3.3C-D). The mRNA levels of both enzymes were each over three times greater in HCASMC compared to HCAEC. Glycosylated ceramide metabolites are often less bioactive than native ceramide. Taken together, HCAEC and HCASMC differentially express mRNAs for ceramide metabolizing enzymes, which may offer an explanation for the dichotomous actions of exogenous C₆-ceramide upon vascular tissues.

3.4.4 Ceramide-1-Phosphate Accumulates in Human Coronary Artery Endothelial Cells

Using HPLC/MS/MS analysis, we next examined if the metabolism of exogenously delivered C₆-ceramide to HCASMC and HCAEC correlates with the differences between sphingolipid enzyme mRNA levels in the two cell lines. Consistent with the higher expression of ceramide kinase mRNA found in HCAEC compared to HCASMC, levels of C-1-P in HCAEC are approximately twice the levels found in HCASMC (Table 3.1) after treatment with C₆-ceramide. Furthermore, following treatment with C₆-ceramide, galactosyl- and glucosylceramide species, quantified together as cerebroside mass, were 6-fold higher in HCASMC (Table 3.1). This correlates well with levels of galactosyl- and glucosylceramide synthase mRNA in HCASMC, which are, as previously mentioned, over 3 times the levels found in HCAEC. Although expression of sphingosine kinase mRNA did not differ significantly between HCAEC and HCASMC, mass levels of mitogenic S-1-P in HCAEC were 50% greater
than levels in HCASMC, further supporting the ability of C₆-ceramide to inhibit SMC growth while promoting re-endothelialization of the wounded artery. No significant mass differences between the cell types were observed for other sphingolipids, including total ceramides, sphingomyelin, and sphingosine (Table 3.1).

Table 3.1 Lipidomic Profile of Basal and C₆-Ceramide-Treated Human Coronary Artery Smooth Muscle Cells and Human Coronary Artery Endothelial Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Type</th>
<th>Total Ceramide (pmol/mg protein)</th>
<th>Ceramide-1-Phosphate (pmol/mg protein)</th>
<th>Cerebrosides (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>HCASMC</td>
<td>382.4 ± 28.5</td>
<td>5.87 ± 0.95</td>
<td>330.50 ± 44.02</td>
</tr>
<tr>
<td></td>
<td>HCAEC</td>
<td>270.6 ± 17.2</td>
<td>4.55 ± 0.88</td>
<td><strong>19.68 ± 4.20</strong></td>
</tr>
<tr>
<td>C₆-Ceramide</td>
<td>HCASMC</td>
<td>8207.8 ± 438.8</td>
<td>9.02 ± 1.30</td>
<td>4070.30 ± 480.60</td>
</tr>
<tr>
<td></td>
<td>HCAEC</td>
<td>7933.4 ± 59.5</td>
<td><strong>17.09 ± 2.67</strong></td>
<td>680.26 ± 36.85</td>
</tr>
</tbody>
</table>

HCAEC bolded, italicized values differ significantly (p<0.05) compared to HCASMC with respective treatment, n = 9-10 separate replicate experiments. Multiple authors contributed to this work. Please see Preface for detailed acknowledgment.

3.4.5 C-1-P enhances VEGF-induced Endothelial Cell Survival

Our studies suggest that HCAEC have the capacity and ability to generate endogenous C-1-P and/or to metabolize exogenously applied ceramide into C-1-P.

Although C-1-P has been shown to be proliferative and/or pro-inflammatory in certain
cell types, its effects on HCAEC have not been validated. Exogenously delivered C₈–C-1-P in liposomal formulations, at a concentration of 1 µmol/L, neither directly induced proliferation in HCAEC nor significantly enhanced VEGF-induced HCAEC proliferation as determined via ³H-thymidine incorporation (Fig. 3.4A). In contrast, exogenously delivered C₈–C-1-P (1 µmol/L) promoted endothelial cell survival by attenuating serum deprivation-induced apoptosis in HCAEC, as determined by caspase 3/7 activity as a marker of apoptosis (Fig. 3.4B). Moreover, under serum-deprived conditions, C₈–C-1-P modestly enhanced VEGF induced endothelial cell survival (Fig. 3.4B). Thus, the physiological correlate of elevated ceramide kinase mRNA expression and elevated C-1-P mass in HCAEC may be diminished apoptotic potential, consistent with enhanced wound healing responses to exogenous ceramide.

3.4.6 C₆-ceramide Modifies the Formation of the Fibrin Matrix

Enhanced fibrinogen deposition and persistent fibrin formation is often associated with thrombus formation as well as a lack of efficient endothelial wound healing responses. Interestingly, surface-adsorbed fibrinogen reportedly initiates the acute inflammatory response to implanted polymers (251). We, thus, evaluated the effects of C₆-ceramide to modify the formation of the fibrin matrix and to regulate the early wound healing response. We examined fibrinogen adsorption to C₆-ceramide coated films and demonstrated that fibrinogen binding was significantly reduced by approximately 35-40% versus the bare film, while a paclitaxel coating had little or no effect (Fig. 3.5).
Figure 3.4 C₈-Ceramide-1-Phosphate (C₈-C-1-P) enhances VEGF induced human coronary artery endothelial cell survival but not proliferation. (A) C₈-C-1-P (1 µmol/L) did not enhance VEGF-induced (10 ng/µL) HCAEC proliferation as determined via ³H-thymidine incorporation, n = 6 separate replicate experiments. (B) C₈-C-1-P (1 µmol/L) attenuated apoptosis (caspase 3/7 activity) in serum-deprived HCAEC. In addition, C₈-C-1-P enhanced VEGF-induced (10 ng/µL) attenuation of apoptosis in serum-deprived HCAEC, n = 5. * Denotes significance compared to serum starved HCAECs. ** Denotes significance compared to serum starved HCAECs treated with VEGF, p=.05.
Figure 3.5 C₆-ceramide modifies the formation of the fibrin matrix. Fibrinogen adsorption to C₆-ceramide-coated films was decreased compared to paclitaxel-coated or bare films. (A) A smaller mass of fibrinogen was adsorbed on C₆-ceramide coated polymer films than on paclitaxel-coated or bare films. (B) Surface-adsorbed fibrinogen mass was used to calculate fibrinogen layer thickness on polymer films according to the Voight model (according to reference (242)), n = 3. Multiple authors contributed to this work. Please see Preface for detailed acknowledgment.
In the next series of experiments, we investigated this putative anti-thrombogenic, negative remodeling mechanism of action for ceramide *in vivo*. We utilized a cholesterol-fed rabbit model of hyperlipidemia. In this model, carotid arteries responded to angioplasty with positive remodeling, thrombosis, and neointimal hyperplasia. We demonstrated that this model has increased levels of cholesterol and LDL, with diminished concentrations of HDL (Fig. 3.6A-C). Moreover, Sudan 4 staining revealed enhanced lipid accumulation within the neointimal and thrombogenic core of the angioplastied carotid arteries (Fig. 3.6D). C₆-ceramide therapeutic balloon treatment reduced the degree of positive remodeling and thrombosis at the site of injury (Fig. 3.6E-G), which correlated with a significant reduction of stenosis (Fig. 3.6I). Dihydro-C₆-ceramide, an inactive ceramide analogue, did not reduce thrombogenesis or stenosis within the damaged artery (Fig. 3.6H). Taken together, the ability of ceramide to modify the formation of the fibrin matrix may regulate the subsequent early healing response and diminish thrombotic events, suggesting an ancillary mechanism by which ceramide contributes to normal healing.
Figure 3.6 C6-Ceramide-therapeutic balloons limit carotid artery positive remodeling, thrombosis, as well as neointimal hyperplasia in a cholesterol-fed rabbit model of hyperlipidemia. A-C) Serum cholesterol (A), triglyceride (B), and HDL (C) levels in rabbits during a 12 week diet of 2% cholesterol, n = 6. Cholesterol and triglyceride levels were elevated with a concomitant decrease in HDL levels. D) Sudan 4 staining revealed that C6-ceramide limited the enhanced lipid accumulation within the neointimal and thrombogenic core of the angioplastied carotid arteries, n = 3. E-I) Representative photomicrographs (E-H) and quantification (I) of arteries from 2% cholesterol fed rabbits show C6-ceramide therapeutic balloon treatment reduced the degree of positive remodeling and thrombosis at the site of injury. Photomicrographs are of a non-angioplastied artery (E), and angioplastied arteries with balloon catheters coated with vehicle (F), C6-ceramide (G), or inactive dihydro-C6-ceramide (H). C6-ceramide coated, but not dihydro-C6-ceramide coated, balloon catheters decreased the post-angioplasty neointimal area of arteries in hyperlipidemic rabbits, n = 6 animals. Multiple authors contributed to this work. Please see Preface for detailed acknowledgment.
3.4.7 C6-ceramide is Non-Toxic in Animal Models

Non-clinical toxicity and pharmacokinetic profiles have been analyzed for C6-ceramide in four single-dose studies (rat and dog), two multiple-dose sub-chronic studies (rat [28 days] and dog [14 days]), and two pharmacokinetic analyses (rat and dog). At doses up to 15 mg/kg in rats and 7.5 mg/kg in dogs there were no drug-related adverse effects based on clinical observations, body weights, feed consumption, hematological parameters, clinical chemistry, electrocardiograms, gross pathology, organ weights, and histopathology (data not shown). There was also no evidence of mutagenicity, assayed by microbial cell mutagenesis, mammalian cell mutagenesis, and the mouse bone marrow micronucleus test with C6-ceramide formulations up to 1000 mg/kg (BioReliance Rockville, MD).

In pharmacokinetic intravenous injection studies, the maximal plasma concentration of C6-ceramide (T\text{max}) was achieved within 2 min of injection. There was good dose proportionality of the maximum plasma concentration of C6-ceramide (C\text{max}) and area under the curve (AUC\text{all}). Elimination from the plasma was biphasic with a short half-life in the distribution phase (around 3-5 min in rats and 4-7 min in dogs) and a longer half-life in the terminal phase (about 1.5 h in rats and between 1.5 - 1.83 h in dogs). Maximum tolerated and therapeutic doses of C6-ceramide were determined for rat and dog; therapeutic ranges of doses were determined for pig and rabbit. We evaluated the non-clinical toxicity of C6-ceramide as it relates to a potential human dose of approximately 150 µg given as a coating on an intracoronary device. Table 2 presents the maximum tolerated C6-ceramide concentrations in two species, the conversion to the human equivalent dose, and the multiple of the expected clinical dose as a measure of the
safety of this compound. In all cases, the expected clinical dose of ceramide used on the therapeutic balloon is three orders of magnitude below the maximum tolerated concentration of ceramide.

**Table 3.2. C₆-Ceramide Doses in Animals and Humans**

<table>
<thead>
<tr>
<th>Species</th>
<th>Maximum Tolerated C₆-Ceramide Concentration</th>
<th>Human Dose Equivalent</th>
<th>Multiple of Expected Clinical Dose (ECD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>15 mg/kg</td>
<td>2.43 mg/kg</td>
<td>972</td>
</tr>
<tr>
<td>Dog</td>
<td>7.5 mg/kg</td>
<td>4.06 mg/kg</td>
<td>1624</td>
</tr>
</tbody>
</table>

* assumes a human clinical dose =150 μg/60kg

Multiple authors contributed to this work. Please see Preface for detailed acknowledgment.

3.5 Discussion

Even though ceramides are usually considered anti-proliferative or pro-apoptotic mediators, tissue specific metabolism can alter the pleiotropic responses to exogenous ceramide analogues. For example, fibroblasts often respond to exogenous ceramide with a proliferative response (216, 252). In the present study, human vascular endothelial cells, in contrast to human vascular smooth muscle cells, responded to ceramide with minimal growth arrest *in vitro* and pro-wound healing responses *in vivo*. The reason for this dichotomy between VSM and endothelial cell responses could be at the level of differential metabolism of exogenously applied C₆-ceramide.

We have now shown that ceramide kinase mRNA expression and C-1-P mass (in C₆-ceramide treated cells) are elevated in HCAEC compared to HCASMC. As opposed to the general anti-proliferative effects of ceramide, C-1-P has been shown to be pro-mitogenic and promote cell survival in several cell types (250). We have extended these
studies to show that C-1-P augmented VEGF induced cell survival in HCAEC. In this way, exogenous ceramide analogues can be preferentially metabolized into ceramide-1-phosphate in endothelial cells, possibly promoting a pro-wound healing response consistent with the lack of pro-thrombotic events in vitro (Fig. 3.5) and in vivo (Fig. 3.6).

It is of interest that the coronary artery endothelial cells contain a higher distribution of the S-1-P receptor (subtype 1) compared to coronary artery smooth muscle cells (80), and that CDases are actively released by murine endothelial cells (113), these findings are consistent with an increased endothelial wound healing response mediated by phosphorylated ceramide metabolites.

Another metabolic difference between HCASMC and HCAEC is at the level of cerebrosides. We have shown that HCASMC have increased expression of glucosyl- and galactosylceramide synthases, correlating with an increased mass of cerebroside species. The role of cerebrosides and/or cerebroside metabolites in VSM is somewhat controversial. Lactosylceramide has been shown to be proliferative in VSM (253) while glucosyl- and galactosylceramide species have been shown to be somewhat less bioactive than ceramide species (55). Regardless, basal human coronary artery smooth muscle cells metabolized exogenous ceramide into these glycosphinogolipid metabolites and not into the highly pro-mitogenic phosphorylated C-1-P and S-1-P species.

The preferential metabolism of ceramide to C-1-P by HCAEC may explain how exogenous ceramide acts as mediator of VSM cell cycle arrest or senescence (107, 108) while simultaneously promoting endothelial cell wound healing responses. Another mechanism that may be responsible for pro-wound healing responses at the site of injury may involve a reduction of fibrinogen absorption/deposition on injured arteries. The
ability of ceramide to modify the formation of the fibrin matrix may contribute to diminished thrombus formation as well as enhanced growth factor- or C-1-P-induced re-endothelialization. In fact, C-1-P has recently been shown to directly activate phospholipase A₂-induced arachidonate release, an important mediator in controlled inflammatory responses such as re-endothelialization or wound healing. Other reports have also commented upon the mechanisms by which ceramide can limit vascular injury. For instance, intravenous or intracisternal delivery of cell permeable ceramide analogues reduces infarct size in SHR rats via induction of tolerance to ischemia (254).

Even though our studies indicate that exogenous ceramide may reduce thrombogenic or fibrogenic events in hypercholesterolemic models, its role in developing atherosclerotic lesions is still somewhat controversial. Ceramide has been shown to be a component of LDL particles (255), yet it is now believed that ceramide metabolites, including S-1-P, lead to foam cell development, atherogenesis, and thrombogenesis (256, 257). In fact, ceramide, itself, may also be beneficial in limiting atherosclerosis through ceramide-dependent eNOS expression (79, 258) and the inhibition of TNF-induced adhesion protein expression (91), subsequent to HDL binding to endothelial scavenger receptors. Ceramide also inhibits gene transcription of sterol regulatory element binding proteins, which mediate a physiological feedback mechanism to lower cholesterol biosynthesis (259). Taken together, the promotion of negative remodeling and/or wound healing are events consistent with the limitation of proliferation, thrombogenesis and fibrogenesis observed in our ceramide-treated hypercholesterolemic rabbit model of arterial injury.
Despite successes with drug eluting stents (DES), limiting restenosis from 20 to 9%, analyses of cost-effectiveness have raised some issues concerning populations and diseases suitable for these devices as well as their ultimate effects upon patient mortality (260). In fact, the Basel Stent Cost Effectiveness Trial (BASKET) study has recently concluded that cost-effectiveness issues should limit DES to elderly populations (>65 years) with lesions greater than 20 mm or arteries thinner than 2.5 mm (261). Moreover, the use of DES for tortuous, disperse, bifurcated, and multiple vascular lesions, especially in diabetes or peripheral arterial disease, may be limited by definition. The fact that ceramide-coated balloon catheters greater than 8 mm limit stenosis in porcine iliac and renal arteries argues for the use of this modality for diffuse injury in large non-coronary beds. Our lipid-based coating process for balloon catheters requires no polymer matrix or x-ray/contrast medium substances, which could augment secondary thrombus formation or inflammatory processes. In addition, the coating process offers a homogeneous surface coating suitable for delivery of hydrophobic substances such as C₆-ceramide. This may offer an advantage over stent coatings where these types of drugs may accumulate within or near stent struts, leading to undesirable toxicological side effects due to the drug or the polymer. Although a single PCTA catheter-administered dose of C₆-ceramide limited stenosis after angioplasty, the temporal aspect of such a treatment renders treatment with drug-coated PCTA catheters at a disadvantage compared to treatment with DES. Inherently, the use of drug-coated PCTA catheters is a short term treatment where one relies on a single dose to exert and maintain its effect, whereas the use of stents (bare or drug-eluting) relies on a more long-term physical apparatus to prevent restenosis. In the case of DES, the applicable drug is delivered for an extended
period of time that could make it a more efficacious delivery method in situations where stent implantation is possible. Advantages of coating conventional PCTA catheters go beyond economical considerations; PCTA catheters offer enhanced flexibility as well as the ability to treat lesions beyond the stent coverage area or to treat in-stent restenosis. These considerations have recently been exploited by the Paclitaxel balloon coating system (240, 241, 262), where inhibition of restenosis can occur independent of chronic delivery via DES implantation (241).

In conclusion, the use of a therapeutic balloon as an adjunct to traditional and drug-eluting stent therapy or as a stand-alone modality has direct clinical applicability and may meet unmet medical conditions associated with pro-inflammatory, pro-mitogenic arterial or venous (SVG) lesions. Moreover, the delivery of an agent that limits neointimal hyperplasia while promoting endothelial wound healing responses directly addresses an important health concern with drug eluting stents, i.e., thrombotic events occurring at the site of stent implantation. The most useful predictor of thrombotic events in drug eluting stents is incomplete reendothelialization (30). Therefore, C6-ceramide, which inhibits stenosis, yet allows reendothelialization, could possibly limit thrombotic events after stent implantation.

The use of C6-ceramide as a therapeutic in preventing stenosis is not without its limitations. We were able to uniformly coat balloon catheters with C6-ceramide in limited numbers for our experiments. However, this procedure is not sufficient for coating large numbers of catheters. The design of procedures to coat catheters or stents with C6-ceramide uniformly and consistently is a limiting factor in using it as a therapeutic. Moreover, applying C6-ceramide to stents is more difficult compared to
balloon catheters and could result in accumulation of C₆-ceramide, which could lead to excessive delivery of ceramide to areas of the artery in contact with portions of the stent containing accumulated C₆-ceramide, possibly leading to apoptosis.
Chapter 4

Overall Discussion, Conclusions, and Future Directions
4.1 Overall Conclusions

Sphingolipids are critical cellular constituents that have diverse functional roles including enhancing membrane integrity and regulating cellular signaling. Maintaining sphingolipid homeostasis is of utmost importance for cells to sustain a normal phenotype, and the negative consequences of altered sphingolipid metabolism is manifested in diseases such as Farber’s Disease, in which a debilitating buildup of ceramide occurs within cells. Sphingolipids can be either beneficial or detrimental to the pathophysiology of many diseases including cancer and atherosclerosis. Thus, the metabolic enzymes that convert one sphingolipid species to another are just as important as sphingolipids themselves. This dissertation provides further insights into possible roles of sphingolipids and sphingolipid metabolism as therapeutics in disease states such as atherosclerosis.

The conclusions presented from the studies within this document focus around the therapeutic use of ceramide and how ceramide metabolism can potentially affect ceramide’s efficacy. We initially chose to investigate the regulation of nCDase (nCDase) because nCDase has crucial roles in regulating the balance between ceramide, sphingosine, and sphingosine-1-phosphate (S-1-P). The balance between ceramide and S-1-P levels within cells has been shown to be crucial in determining cellular fate; altering nCDase activity has the potential to send a cell from a death pathway to a survival pathway or vice versa, thus understanding nCDase regulation could aid in the design of therapeutic treatments or adjuncts. No work was previously done on the transcriptional regulation of nCDase, so we initiated our studies by identifying the proximal promoter of the nCDase gene and demonstrating that there are likely multiple
activators and repressors that prevent large changes in nCDase expression. The proximal promoter was activated by serum which correlated with serum-induced increases in nCDase mRNA expression. Several functional transcriptional response elements (TRE) were identified within the proximal promoter, including binding sites for AP-1, NF-Y, Oct-1, AP-2, and GATA factors. Each TRE was necessary for maximal promoter activity. However, the overall activity of the nCDase promoter was not dependent on any one TRE, suggesting that the transcriptional activity arises from concerted and combinatorial interactions of multiple transcription factors. In addition, overexpression of AP-1, a serum-activated transcription factor, was sufficient to increase promoter activity. In contrast, knocking down AP-1 with siRNA lowered the maximal promoter activity indicating that AP-1 is one transcription factor that works concertedly with other transcription factors to regulate nCDase transcription.

With a basic understanding of nCDase transcriptional regulation, we next investigated nCDase in an in vivo model. Specifically, we were interested in its regulation in the vasculature because initial studies with smooth muscle cell pericytes indicated that ceramide potentially had anti-stenotic properties (234), which could possibly be altered by nCDase because of its role in converting ceramide to S-1-P. Cell permeable, synthetically derived C₆-ceramide was able to drastically inhibit stenosis and internal elastica injury in large arterial beds. With in vitro models, C₆-ceramide significantly reduced proliferation of human coronary artery smooth muscle cells (HCASMC) but had minimal effects on the growth arrest of human coronary artery endothelial cells (HCAEC). Similar effects were demonstrated in vivo when C₆-ceramide limited vascular smooth muscle cell (VSMC) hyperplasia but permitted
reendothelialization in healing of the wounded endothelium after angioplasty-induced stretch injury. The mRNA levels of various sphingolipid metabolizing enzymes in HCASMCs and HCAECs were analyzed, and the analyses did not detect any significant differences in nCDase mRNA expression between the two cell types. Interestingly, these results are not that surprising when one considers how tightly regulated nCDase transcription may be. However, these findings do not preclude nCDase from being involved in the etiology or treatment of atherosclerosis. Surprisingly, HCAECs expressed relatively higher levels of ceramide kinase mRNA compared to HCASMCs, which correlated with increased levels of C₆-ceramide-1-phosphosphate (C₆-C₁-P) in HCAECs compared to HCASMCs after C₆-ceramide treatment. C₁-P potentially promotes endothelial cell survival as treatment with exogenously administered C₁-P attenuates serum-deprivation-induced apoptosis in HCAECs. Future experiments expanding our studies will be explained in the following two sections.

4.2 Future Directions: Transcriptional Regulation

To understand the transcriptional regulation of nCDase, we first identified the proximal promoter, which had maximal transcriptional activity, and then used software analysis to determine putative TREs within this proximal promoter. We subsequently demonstrated that the putative TREs are functional and can bind their respective transcription factors; however binding assays were done in vitro using cell nuclear extracts. Further studies are necessary to determine if transcription factors bind the putative TREs in vivo. The binding of transcription factors to cis-elements in vivo can be assessed using chromatin immunoprecipitation (ChIP) assays, whereby bound
transcription factors are crosslinked to the DNA in whole cells before the DNA/protein complex is collected, separated, and analyzed.

Results from in vivo and in vitro studies can be extended to determine the roles of identified transcription factors in biological responses. We demonstrated that overexpression of AP-1 was sufficient to increase transcriptional activity of the human nCDase proximal promoter using reporter assays and that siRNA-mediated knockdown of AP-1 decreased the activity. Similar experiments can be done with the corresponding trans-activators that bind other identified cis-elements. We would then need to demonstrate whether altering AP-1 or other transcription factors would directly affect nCDase transcription within cells by measuring nCDase mRNA levels using quantitative RT-PCR and/or northern blot analyses. Furthermore, any observed changes in nCDase expression would be correlated with the effects of transcription factor alteration on nCDase enzymatic activity. Because CDase is a key enzyme in regulating the balance between pro-apoptotic ceramide and pro-mitogenic S-1-P, the effects on apoptosis and proliferation in response to altering nCDase transcription through the manipulation of trans-activating factors will also be explored.

Although we found multiple functional TREs within the proximal promoter, mutation of any one binding site was not sufficient to abrogate total promoter activity. As each cis-element did contribute to the total maximum promoter activity, we concluded that promoter activity was based on combinatorial interactions of multiple transcription factors. Future experiments will be performed to determine which combination of transcription factors give rise to the majority of transcription potential of the human nCDase proximal promoter. Such experiments will explore what effects simultaneous
mutation of multiple TREs will have on the maximal promoter activity. In addition, the activity of a promoter construct that has all identified TREs mutated will be assessed to determine if we failed to identify a pertinent cis-element within the proximal promoter.

Once the combinatorial interactions of transcription factors that regulate the human nCDase proximal promoter have been elucidated, analysis of the rest of the nCDase 5’-untranslated region can commence. We demonstrated that the DNA extending 5’ of the proximal promoter most likely contains repressor elements as the transcriptional activity drastically declines over approximately 2,500 base pairs (bp). Similar experiments as described in chapter 2 will be performed to localize and identify any repressor elements in the extended 5’-UTR. Identification of such repressors could attest the fact that serum, a powerful inducer of gene transcription, induced a relatively small increase in nCDase transcription. In addition, the base pair region beyond 2,500 bp upstream of the translational start site will be characterized because that region restores some of the activation potential lost between the proximal promoter and itself.

As CDase is a rate-limiting enzyme in the conversion of ceramide to pro-mitogenic S-1-P, it is plausible that mitogens such as growth factors should activate nCDase. In fact, initial studies suggested that nCDase was activated by PDGF-ββ in renal mesangial cells (133). In our initial studies characterizing human nCDase transcriptional regulation, we used serum as an inducer of transcription because it is a broad encompassing mitogen. However, under the powerful mitogenic stimuli from serum, we could only obtain less than a two-fold induction of nCDase mRNA. This could be attributed to the fact that the levels of sphingosine and S-1-P are an order of magnitude less than ceramide. Thus, small changes in sphingosine or S-1-P can have
drastic cellular effects, so much so that any greater changes in CDase expression could adversely disrupt the sphingolipid balance. On the other hand, it is possible that nCDase transcription is not strongly upregulated by growth factor-type signaling. In support of this argument, some studies were unable to detect activation of nCDase in mesangial cells after administration of platelet-derived growth factor-ββ (PDGF-ββ) (138).

These immediately preceding studies have also implicated nCDase as being upregulated by inflammatory cytokines and as having important roles for cell survival in response to such cytokines. For instance, the cytokine interleukin-1β (IL-1β) increased nCDase activity in rat hepatocytes in a bimodal manner (136). In addition, another cytokine, tumor necrosis factor-α (TNF-α), was reported to cause a delayed activation of nCDase in mesangial cells (263). In a similar delayed manner, IL-1β also stimulated nCDase activity in mesangial cells. Furthermore, IL-1β and TNF-α induced nCDase activity in insulin-secreting rat pancreas cells (264). IL-1β and TNF-α are both potent inflammatory cytokines that actively participate in many inflammatory diseases, including atherosclerosis, and, in fact, their effects are often synergistic with each other. An increase in nCDase activity appeared to protect mesangial cells against IL-1β as inhibiting the increase in nCDase activity resulted in IL-1β inducing DNA fragmentation, an early sign of apoptosis (137). NCDase also protected against TNF-α-induced hepatocyte death, whereby overexpression of human nCDase in rat liver or hepatocytes attenuated TNF-α hepatotoxicity in vivo and in vitro, respectively (135). Evidence suggests cytokine activation of nCDase arises, at least partially, from induction of nCDase gene transcription. In mesangial cells, IL-1β induced transcription of nCDase mRNA, which peaked at four hours but persisted through 24 hours (137). Moreover, IL-
1β and TNF-α increased nCDase mRNA levels in rat pancreas cells over a period of 4 to 24, peaking at 12, hours (264).

Activation of nCDase transcription by TNF-α is not unexpected as TNF-α has been shown to turn on many genes in inflammatory signaling cascades by activating JNK or other kinases that increase c-Jun/AP-1 activity. For example, TNF-α activated c-Jun N-terminal kinase (JNK) and, subsequently, AP-1, leading to transcriptional activation of ICAM-1 and VCAM-1 in vascular endothelial cells (265). Also, activation of AP-1 by p38-mediated TNF-α stimulation induced transcription of matrix metalloproteinase-9 in multiple types of cancer cells (266, 267). However, the mechanisms behind TNF-α regulation of nCDase have not been investigated. Since TNF-α plays a critical role in the pathogenesis of atherosclerosis, our characterization of nCDase transcriptional regulation may provide insights into the possible role of TNF-α-induced nCDase expression in atherosclerosis. Interestingly, we showed that c-Jun was an important transcription factor in regulating the transcriptional activity of the human nCDase proximal promoter. Therefore, we speculate that TNF-α may induce nCDase transcription through activation of AP-1 and stimulation of the AP-1 response element in the human nCDase promoter. A major issue with this rationale is that all work demonstrating cytokine activation of nCDase mRNA was done in rat cells, whereas we characterized the promoter for human nCDase. Although the rat nCDase promoter is unknown, the mouse nCDase promoter was characterized (216), but we were unable to identify any conserved TRE between the human and mouse promoter. Once we demonstrated that TNF-α could induce human nCDase transcription, we could then proceed to test the role of AP-1 in its transcription.
Moreover, while identifying critical enhancer or repressor TREs in the distal promoter region, we would focus on the response element for another transcription factor linked to TNF-α signaling, i.e., NF-kappaB (NF-κB). NF-κB is a ubiquitous, inducible transcription factor that activates enhancers in a plethora of genes. We are particularly interested in NF-κB because it is a main transcription factor, in addition to AP-1, that is activated through TNF-α signaling. In endothelial cells, NF-κB was reported to be a critical factor for TNF-α-induced transcription of several genes including cell adhesion molecules (268). Furthermore, NF-κB enhancer sites in genes have been found up to several thousand bp upstream of the start site (269), so if TNF-α can also induce human nCDase expression, NF-κB would be a logical enhancer element to look for distal to the proximal promoter.

The mechanism underlying IL-1β activation of nCDase transcription needs to be explored also. IL-1β was shown to potently activate the p38 MAPK pathway in mesangial cells (137). Stimulating p38 can lead to the activation of multiple transcription factors including CHOP/GADD153 (270), which is a member of the CAAT/enhancer-binding protein family of transcription factors. This may have important implications in the regulation of human nCDase gene transcription, as one of the important cis-elements that we identified was a CCAAT box. We showed that the CCAAT box bound with the transcription factor NF-Y, but we did not explore if CHOP/GADD153 bound to this CCAAT box. If CHOP/GADD153 was found to bind to the CCAAT box within the human nCDase promoter, then its role in the activation of nCDase transcription could be explored similarly to as described above, with the listed limitations still applicable.
addition, p38 has been shown to activate AP-1 also (266), so both TNF-α and IL-1β could possibly induce transcription of nCDase via AP-1 activation.

An understanding of the regulation of nCDase and other sphingolipid metabolizing enzymes will not only help elucidate the roles of sphingolipids in the pathophysiology of disease, but it could also aid in the design of therapeutics and the explanation of differential responses to therapeutics. Altered sphingolipid metabolism has been implicated as a contributor to a wide range of diseases. For instance, sphingosine kinase was found to be overexpressed in multiple types of cancers, including, but not limited to, breast, colon, lung, ovarian, and brain cancers (271, 272, 273). The links between sphingolipid metabolism and obesity are demonstrated by increases in sphingomyelinase (SMase) and serine palmitoyl transferase (SPT) levels in adipose tissue of obese mice and, also, by higher levels of sphingomyelin and ceramide found in serum of obese mice (274). Irregularities in sphingolipid metabolism contribute to multiple disease of the brain such as Alzheimer’s disease where increased levels and activities of acid CDase and acid SMase correlated with elevated ceramide and sphingosine levels in brain tissue from Alzheimer’s patients (275, 276, 277). In addition, evidence collected over the past few years demonstrates a role of sphingolipid metabolism in lung disorders, implicating sphingolipid metabolism as a potential therapeutic target for multiple lung diseases such as emphysema, asthma, and cystic fibrosis (278).

4.3 Future Directions: Analysis of Sphingolipid Metabolism In Vivo

The work presented here and previous work in our lab (107, 108) clearly demonstrated that exogenous C6-ceramide mediated VSMC cell-cycle arrest and, thus,
was able to inhibit neointimal hyperplasia in response to stretch injury in multiple animals and arterial beds. The growth inhibitory effects of ceramide on VSM cells support the overall body of evidence that ceramide is growth-arresting and/or pro-apoptotic (49). In limited cell types, there have been instances where ceramide was reported to exert opposing cellular effects, e.g., ceramide induced proliferation of a fibroblast cell line (252). Although not in opposition to consensual effects of ceramide, our current studies demonstrated that vascular endothelial cells were less susceptible to the growth inhibitory effects of ceramide compared to VSM cells.

Although we have demonstrated a therapeutic efficacy of ceramide on preventing stenosis in vivo and differential metabolism of ceramide by HCASMCs and HCAECs in cell culture, we have not explored the metabolism of ceramide in vivo after balloon angioplasty stretch injury. It would be of interest to see if porcine vascular ECs have an enhanced capacity to metabolize angioplasty-administered ceramide into C-1-P or other anti-apoptotic/anti-growth arresting sphingolipids compared to their VSMC counterparts. Experiments testing these effects are not without their limitations but recent advances in techniques and instrumentation make it possible to study sphingolipid metabolism in vivo. For such studies, the endothelium and VSMCs must be isolated separately subsequent to C₆-ceramide coated balloon catheter angioplasty. Separation of the vascular ECs from VSMCs could be performed similar to the isolation of ECs from canine coronary arteries done by Dame et al. (279). Basically, in such a procedure, a weak collagenase solution is incubated in the artery that is only strong enough to detach the endothelium. After removal and collection of the endothelium, a collagenase and elastase solution is used to detach and recover the VSM cells. The sphingolipids would
then be extracted from the isolated ECs and VSMCs and analyzed by mass spectrometry to determine how \( \text{C}_6 \)-ceramide was metabolized. A major limitation of such an experiment would be acquiring enough vascular ECs to obtain sufficient amounts of lipids to be detected by the mass spectrometer. However, continued advances in mass spectroscopic techniques such as electrospray ionization are constantly increasing the sensitivity in detecting sphingolipid levels, which should permit the analysis of low sample amounts (1).

The sphingolipid metabolic enzyme profile in arterial cells was not characterized in our porcine \textit{in vivo} model either. Isolating the ECs and VSMCs from the treated arteries would also allow us to quantify the relative levels of mRNA in each cell type for the various sphingolipid metabolizing enzymes. Completely pure samples of vascular ECs and VSMCs can be obtained from the original collection samples by using fluorescence activated cell sorting (FACS) flow cytometry with markers specific for vascular EC or VSM cells similar to that done by Oxhorn et al. (280). The samples collected from the FACS analysis would give the necessary 100% pure sample of cells to perform multi-cell RT-PCR. Multi-cell RT-PCR, as performed by Gauthier et al., would allow for the quantification of sphingolipid metabolic enzyme mRNAs from a cell sample containing as little as 10-15 cells (281). Of note, there is a lack of clean antibodies raised against sphingolipid metabolizing enzymes, which limits experiments analyzing actual enzyme levels. Hopefully, this will be resolved in years to come.

Any \textit{in vivo} lipodomic profile and/or sphingolipid metabolic enzyme comparison obtained from experiments described above would hopefully support our results that demonstrated the differential response to ceramide between VSMCs and vascular ECs in
both *in vivo* and *in vitro* experiments. The (over)expression of sphingolipid enzymes has been shown to minimize the effects of ceramide in multiple cell types, both positively and negatively. Metabolism of ceramide into non-apoptotic sphingolipids is often increased in cancer cells. Accordingly, several drug-resistant tumor cell lines were shown to express higher levels of glucosylceramide synthase and have higher levels of glucosylceramide after treatment with ceramide-releasing chemotherapeutic drugs compared to respective drug-sensitive tumor cells (282). Furthermore, pharmacological and molecular inhibition of glucosylceramide synthase in adriamycin-resistant breast cancer cells sensitized the cells to adriamycin and other chemotherapies (283). In normal cells, the upregulation of sphingolipid metabolizing enzymes can help protect against cellular stress and cytokine release. As previously mentioned, nCDase expression was upregulated in mesangial cells and pancreatic beta cells in response to the ceramide-releasing cytokines IL-1β and TNF-α, respectively, thus protecting the cells from ceramide-induced apoptosis (137, 264). Future experiments will explore if levels of nCDase are increased in cancer cells that are resistance to ceramide-induced apoptosis and, also, test methods of regulating nCDase expression in order to increase cancer cell sensitivity to ceramide.

Because of the above data and the crucial involvement of CDase in converting ceramide to strongly mitogenic/anti-apoptotic S-1-P, we originally hypothesized that vascular ECs may express relatively higher levels of nCDase compared to VSMCs, thus evoking the ceramide-insensitive phenotype manifested by vascular ECs. Surprisingly, our analyses did not reveal any significant differences in nCDase levels or sphingosine and S-1-P levels in response to administration of ceramide between the two cell types.
However, our results indicated that HCAECs express significantly higher levels of ceramide kinase (CERK) and metabolize more of the administered ceramide into ceramide-1-phosphate (C-1-P) compared to HCASMCs. This was a very surprising, albeit interesting, finding. Studies on CERK and C-1-P are relatively new and have only begun to accelerate in growth over the past few years. Most data suggest that C-1-P may regulate proliferation, cell motility, cell survival, or inflammation. Increasing evidence emphasizes the role of CERK and C-1-P in inflammation. C-1-P was reported to be an activator of phospholipase A2, the rate-limiting enzyme in the synthesis of arachidonic acid and subsequent inflammatory eicosanoid synthesis (284). In addition, C-1-P has been shown to increase proliferation and migration and, also, inhibit apoptosis in macrophages (285, 286, 287), which are key regulators of inflammation in atherosclerosis. New data from our lab further support a role for C-1-P in inflammation and atherosclerosis. These data demonstrated that lipopolysaccharides (LPS), inflammatory agents that activate Toll-like receptor-4 (TLR4), increases CERK mRNA and protein levels in rat aortic SMCs (unpublished data; Houck, Fox, O’Neill, Kester). Increases in CERK correlated with an increase in C-1-P mass with LPS stimulation (unpublished data; Houck, Fox, O’Neill, Kester). Moreover, administration of C-1-P induced in vitro markers of atherosclerosis including VSMC proliferation, positive remodeling, and production of inflammatory cytokines (unpublished data; Houck, Fox, O’Neill, Kester). Increases of CERK and C-1-P in response to LPS can be correlated with the ability of LPS to accelerate the formation of atherosclerotic lesions in hypercholesterolemic rabbits (288). Although it can be inferred from these data that the conversion of ceramide to C-1-P would not be beneficial to the treatment of
atherosclerosis, the enhanced capacity of vascular ECs to metabolize ceramide into C-1-P, but not VSMC, may actually be anti-atherogenic.

From these results, HCAECs’ resistance to ceramide was attributed to increased levels of ceramide kinase and their enhanced capacity to metabolize the exogenously administered ceramide into C-1-P. Both ceramide kinase and its product, C-1-P, have been implicated in inducing proliferation in multiple cell types (250, 289, 290). In our studies, we were unable to demonstrate that C-1-P could induce proliferation in HCAECs. However, we did show that C-1-P protected the HCAECs from serum-deprivation-induced apoptosis. A similar effect was seen in human keratinocytes where C-1-P also protected against serum induced apoptosis (291). Hence increased levels of ceramide kinase within cells, as vascular ECs have, can potentially shift the balance of pro-apoptotic ceramide to anti-apoptotic C-1-P.

An increased potential for vascular ECs to metabolize ceramide into anti-apoptotic sphingolipids like C-1-P could be a mechanism that helps protect against the development of atherosclerosis. Disruption of the vascular endothelium is thought to be an initial step in the pathogenesis of atherosclerosis (10); as such, protection against growth arrest and/or apoptosis in response to cytokines or other ceramide-releasing agents may limit the degree of endothelium dysfunction. Similarly, resistance of vascular EC to the growth inhibitory effects of ceramide actually makes the therapeutic use of C₆-ceramide an attractive alternative in the prevention of restenosis. When using drug-eluting stents to prevent restenosis, incomplete reendothelialization is a primary predictor of thrombotic events (29, 30). Because ceramide can inhibit VSMC growth while allowing the wounded endothelium to heal after balloon angioplasty, the use of ceramide
in drug eluting stents could possibly promote reendothelialization and, hence, limit post-
angioplasty thrombotic events. Interestingly, we also demonstrated that the two drugs
that are currently used clinically in drug-eluting stents, rapamycin and paclitaxel (26),
both inhibit HCAEC proliferation, which could hinder reendothelialization.

C-1-P may also have other potential anti-atherogenic or anti-stenotic effects,
possibly by promoting endothelial cell migration. The migratory effects of C-1-P were
clearly demonstrated in a macrophage cell line, and evidence strongly suggests these
effects were mediated through a G\textsubscript{i} protein-coupled receptor that is distinct from S-1-P
receptors (287). C-1-P-induced migration in the macrophages was dependent on both
MEK/ERK and PI3K/AKT pathways, or more precisely, the p110\textsubscript{\beta} isoform of PI3K
(290). Interestingly, p110\textsubscript{\beta} was very recently found to signal downstream of G-protein-
coupled receptors in vascular ECs (292). With the appropriate stimulation, vascular ECs
can be extremely migratory, which is biologically necessary for maintaining endothelium
integrity and for angiogenesis. It is possible that p110\textsubscript{\beta} in vascular endothelial cells
could signal downstream of the putative C-1-P receptors. Until such receptors are
identified and cloned, the effects of C-1-P on HCAEC and, for comparison, HCASMC
migration could be explored in a manner similar to that described by Granado et al. (287).
Once (a) specific C-1-P receptor(s) is(are) identified, it would be interesting to
characterize and compare receptor expression patterns between HCAEC and HCASMC
to determine if expression differences could explain any differential responses to C-1-P
between the two cell types. The importance of such studies is exemplified by the effects
of S-1-P, which tends to have pro-atherogenic effects on VSM cells, but anti-atherogenic
effects on vascular ECs. This phenomenon arises, at least in part, from VSM cells
expressing the S1Pr-2 (S-1-P receptor 2) isoform predominantly (80), whereas vascular ECs express S1Pr-1 and S1Pr-3 isoforms (83). Therefore, the response of a cell type to a particular agent, e.g., C-1-P, may depend on receptors that the cells express. If multiple receptors exist, they may be coupled to different G proteins that could account for diverse effects of C-1-P. The degree to which ECs secrete C-1-P is also important to determine if the ability of HCAEC to convert ceramide to C-1-P would lead to EC migration. If C-1-P-induced migration is truly receptor-mediated, the C-1-P must be outside the cell to reach cell surface receptors as Granado et al. demonstrate (287). Although this group did not test the ability to secrete C-1-P, C-1-P was reported to be readily secreted by blood monocytes and, possibly, by other cell types (293). It is feasible that vascular ECs could secrete C-1-P as they have been shown to secrete CDases and SMases (113, 294). It is also possible that there may be no C-1-P receptors and that signaling occurs through intracellular targets of C-1-P, which is very conceivable even if C-1-P receptors exist.

Data from our lab actually indicates that mTOR is an intracellular target of C-1-P (unpublished data; Fox, Ropson, Kester). Whatever the case may be, C-1-P and CERK are important targets of future studies identifying the role of sphingolipid metabolites and sphingolipid metabolism in the pathogenesis and treatment of atherosclerosis.

4.4 Model and Concluding Remarks

Regardless of the specific metabolism of the administered C6-ceramide, the work presented within this dissertation and previous work done in our lab clearly demonstrate that C6-ceramide is able to limit stenosis after arterial stretch-injury by inhibiting vascular smooth muscle cell growth. In addition, C6-ceramide appears to attenuate the damage to the endothelium after angioplasty. However, the data in this dissertation does not directly
address the mechanisms on how C₆-ceramide is affecting the vascular cells. Here we propose a four part mechanism of action model by which C₆-ceramide reduces stenosis after arterial stretch injury and discuss how relevant each part may be to attenuating stenosis after injury. The first part hypothesizes that the viscoelastic properties of ceramide may reduce the degree of injury inflicted upon the arteries after angioplasty. Secondly, a change in total lipid mass after C₆-ceramide treatment may result in inhibition of VSM cell growth and subsequent decrease in stenosis. The third part of the model suggests the observed effects are a result of C₆-ceramide activating growth arresting or apoptotic signaling cascades. Finally, we propose that C₆-ceramide may inhibit VSM cell growth through intercalating into and disrupting the membrane.

Although they are part of the model, two of the above mechanisms of action likely contribute only minutely to the observed effects of C₆-ceramide, as determined from results of negative controls. The viscoelastic hypothesis takes into consideration that C₆-ceramide is a lipid, and as a lipid, it could act as a lubricant when it is applied to balloon catheters. Under this assumption, the C₆-ceramide-“lubricated” catheter would not be as injurious to the artery during angioplasty. A decreased coefficient of friction on the balloon could limit the amount on endothelial denudation during the rotation of the inflated catheter within the artery. As a control for C₆-ceramide, catheters were also coated with C₆-dihydroceramide, a relatively biologically inert sphingolipid differing from C₆-ceramide only by the lack of the 4-5 trans double bond. Therefore, C₆-dihydroceramide should function as a lubricant very similar to how C₆-ceramide functions. However, C₆-dihydroceramide was not able to inhibit stenosis, suggesting that the ability of C₆-ceramide to act as a lubricant had little to do with its biological effects.
The inability of C6-dihydroceramide to inhibit stenosis after stretch injury also suggests the second part of our model may not contribute significantly to explaining the effects of C6-ceramide on stenosis. The second hypothesis suggests that VSM cell growth is inhibited by the large change in total lipid mass within the cells after they are administered the C6-ceramide. Indeed, the amount of ceramide, i.e. lipids (sphingolipids), taken into the cells after treatment is much higher than physiological levels of sphingolipids within the cells. An overall buildup of sphingolipids can lead to dysfunctional or apoptotic cells, as clearly demonstrated in Drosophila Sply05091 mutants that lack functional S1P lyase, the enzyme catalyzing the major exit point of sphingolipid metabolism (295). These mutants accumulate sphingolipid metabolites leading to multiple abnormalities including increased cellular apoptosis (295). Because equivalent amounts of ceramide and dihydroceramide were used on catheters in angioplasty, yet yielded different results, it is unlikely that the effects of C6-ceramide on stenosis can be attributed to an overall change in sphingolipid mass within cells.

The third and fourth parts of our model both could rationally explain the results we observed. The effects of C6-ceramide on stenosis after stretch injury more than likely arise from a combination of mechanisms underlying parts three and four of our proposed model. The first of these suggests that ceramide is a second messenger in cell signaling and leads to activation or inhibition of downstream effectors. Previous work from our lab demonstrated that C6-ceramide decreased AKT and ERK activation in rabbit VSM cells after angioplasty, which correlated with a C6-ceramide-induced decrease in stenosis in stretch-injured rabbit carotid arteries (107). PKCζ has been shown to be activated by ceramide, and ceramide-activated PKCζ was necessary for C6-ceramide-induced
inactivation of AKT and inhibition of VSM cell proliferation (108). Interestingly, C₆-
dihydroceramide did not inactivate AKT or decrease VSM proliferation. These data
support the role of ceramide as a second messenger in the inhibition of stenosis after
arterial stretch injury.

The fourth part of the model accounts for membrane disruption as a cause for the
observed reduction of restenosis after angioplasty with C₆-ceramide coated catheters.
There is much evidence in the literature supporting that ceramide exerts some of its
effects by directly intercalating into membranes and disrupting the membranes. Both
short chain (C₂ and C₆) and long chain (natural) ceramides are able to increase membrane
permeability (296) by self-aggregating and forming large ceramide channels within cell
membranes, thus increasing the conductance of the membranes and possibly leading to
apoptosis (297). The formation of the ceramide channels was demonstrated to be
concentration dependent; hence, larger doses of ceramide would lead to formation of
larger channels and, subsequently, greater membrane disruption (297). Interestingly,
both short and long chain dihydroceramides were not able to able to form ceramide
channels or induce apoptosis (297), correlating with our observations that C₆-ceramide,
but not C₆-dihydroceramide was able to reduce stenosis after arterial stretch injury. In
addition, the use of C₆-ceramide in this model offers the advantage of using a membrane
permeable ceramide as opposed to “natural” ceramides, which do not translocate through
membranes and, in fact, have limited mobility from the side of the bilayer that they are
generated in, or administered to, to the other half of the bilayer (298, 299). Because of
their permeability, short chain ceramides, including C₆-ceramide, are able to leave the
plasma membrane and translocate to other membranes inside the cells (300, 301). The
permeated ceramide can intercalate into organelle membranes, including the mitochondrial membrane where the ceramide can induce cytochrome \(c\) release by forming ceramide channels and increasing mitochondrial membrane permeability (296, 297, 302). Disruption of mitochondrial membranes and release of cytochrome \(c\) can lead to apoptosis, providing an additional support for membrane disruption contributing to the ability of \(C_6\)-ceramide to inhibit stenosis. According to the evidence presented above on our proposed mechanism of action model and from the data we have observed in our studies, it appears that the ability of ceramide to act as a second messenger in activating or inactivating downstream effectors and its ability to intercalate and disrupt cellular membranes may be the major underlying mechanisms behind the \(C_6\)-ceramide-induced reduction of stenosis after PCTA balloon catheter-induced stretch injury.

Comprehensively, the studies within this dissertation were done to advance our knowledge of sphingolipid metabolism and its broad application to disease pathogenesis and drug development. In applying sphingolipid metabolism to disease, attention was focused on both atherosclerotic disease progression and on the development of a therapeutic inhibitor of restenosis. As deregulation of sphingolipid metabolism is manifested in many disease states, an understanding of the transcriptional regulation of human nCDase acquired from this work offers a target for the development of drugs that modulate ceramide metabolism. In addition, our studies give promise in the development of novel sphingolipid-based mimetic inhibitors of restenosis that take advantage of differential ceramide metabolism between arterial cell types, which may circumvent complications arising from current drugs contained in drug-eluting stents in surgical treatment of atherosclerosis.
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